Membrane-bound Na,K-ATPase: Target Size and Radiation Inactivation Size of Some of Its Enzymatic Reactions

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Frozen samples of membrane-bound pig kidney Na,K-ATPase were subjected to target size analysis by radiation inactivation with 10-MeV electrons at \(-15^\circ\text{C}\). The various properties investigated decreased monoexponentially with radiation dose, and the decay constants, \(\gamma\), were independent of the presence of other proteins and of sucrose concentrations above 0.25 M. The temperature factor was the same as described by others. Irradiation of four proteins of known molecular mass, \(m\), showed that \(\gamma\) for protein integrity was proportional to \(m\) with a proportionality factor about 20% higher than that conventionally used.

By this standard curve, glucose-6-phosphate dehydrogenase activity used as internal standard gave a radiation inactivation size of 110 \(\pm\) 5 kDa, very close to \(m = 104-108\) kDa for the dimer, as expected. For Na\(^{+}\)/K\(^{+}\)-transporting ATPase the following target sizes and radiation inactivation size values were very close to \(m = 112\) kDa for the \(\alpha\)-peptide: peptide integrity of \(\alpha\), 115 kDa; unmodified binding sites for ATP and vanadate, 108 kDa; \(K^+\)-activated \(p\)-nitrophenylphosphatase activity, 106 kDa. There was thus no sign of dimerization of the \(\alpha\)-peptide or involvement of the \(\beta\)-peptide. In contrast, optimal Na\(^{+}\)/K\(^{+}\)-transporting ATPase activity had a radiation inactivation size \(= 180 \pm 7\) kDa, and total nucleotide binding capacity corresponded to 72 \(\pm\) 3 kDa. These latter results will be extended and discussed in a forthcoming paper.

Thirty years after the discovery of the membrane-bound Na\(^{+}\)/K\(^{+}\)-transporting ATPase (EC 3.6.1.37) (Skou, 1957), its structural and functional organization in the plasma membrane is still subject to controversy. Since this enzyme is identical to the Na,K-pump that couples hydrolysis of ATP to active, outward transport of Na\(^{+}\) and inward transport of K\(^{+}\), its membranal subunit structure and assembly are obviously very important problems.

The enzyme has two subunits, the \(\alpha\)-peptide (\(m = 112\) kDa, Shull et al., 1985) and the \(\beta\)-peptide (\(m = 35\) kDa, Shull et al., 1986), that copurify and cosolubilize after mild detergent treatment, but the molar \(\beta/\alpha\) ratio in the membrane is not known with certainty. Assuming stoichiometric association the ratio is presumably 1, but it could be 2, and it may depend upon the enzyme source (Jørgensen, 1982). Recent reviews reveal, furthermore, that not only is the structural association in the membrane of \(\alpha\) and \(\beta\) between \(\alpha\)-peptides and \(\beta\)-peptides poorly characterized (Reynolds, 1988), but the size of the functional unit ("i.e. the minimal assembly of structures required to give the measured function ...", Steer et al., 1981) and the possible kinetic subunit interactions are also ill defined (Askari, 1988).

The catalytic peptide with the substrate site is \(\alpha\), whereas the functional role of \(\beta\) is unknown. Its role may be in sorting, transporting, and inserting \(\alpha\)-peptide in the plasma membrane (Takeyasu et al., 1988). A key problem is whether an \(\alpha\)-peptide chain is a sufficient structural and functional entity or whether dimers or oligomers of the catalytic peptide (possibly in association with \(\beta\)-peptides) are the minimal functional units for Na,K-ATPase and Na,K-pumping activity. This question seems to be general for several ion-transport systems (Nørby, 1987; Andersen and Vilsen, 1988).

In the present communication, we have studied one of the few methods that have been used to characterize the in situ structural and functional assembly of membrane proteins. Irradiation of biological samples in the frozen or freeze-dried state with high energy electrons leads to loss of primary structure and biological function. Measurement of these parameters as a function of radiation dose will according to classical target size analysis give information on the mass of the structural and functional units of soluble as well as membrane-bound proteins and enzymes (Jung, 1984; Harmon et al., 1985; Beauregard et al., 1987). The method has been employed on a variety of transport ATPases, including Na,K-ATPase, and also on the Cl\(^{-}\) carrier. The results are generally assumed to be compatible with a dimeric arrangement of the active subunit also in cases where other approaches suggest a monomer as the minimal functional unit (see Nørby (1987) for a review). One notable exception, however, is the report by Karlish and Kempner (1984) in which the target sizes of Na,K-ATPase and Na,K-pump activity were found to be smaller than the size of an \(\alpha\)-dimer. Intriguing are also the many observations that the "partial reactions" have smaller radiation sizes than the "total" Na,K-ATPase reaction (Glynn, 1985; Cavieres, 1987).

We have reinvestigated the radiation inactivation method with special regard to its versatility in characterizing the in situ assembly of Na,K-ATPase. The method is not without problems (Harmon et al., 1985; Beauregard et al., 1987) and to be useful for our purpose all the procedures must be rigorously controlled and highly reproducible. Sufficient to mention that to discriminate between molecular masses of, for example, \(\alpha\) and \(\alpha\beta\), \(\alpha\beta\) and \(\alpha\beta\), or \(\alpha\beta\) and \(\alpha\beta\beta\), an accuracy considerably better than \(\pm 10\%\) is required. Just as important is the absolute value of the factor(s) by which the radiation
inactivation "decay constants" (see "Results") are converted to equivalent molecular mass, the target size or the radiation inactivation size, RIS (Beauregard et al., 1987).

Apart from a number of precautions to minimize secondary effects of radiation and to maintain sample stability after irradiation, we have used two main approaches to avoid systematic errors and variability. First, we have produced a standard curve for the conversion of decay constants to equivalent molecular mass. The curve is based on irradiation of four proteins (three enzymes) with known molecular mass, under exactly the same circumstances as those used for irradiation of the Na,K-ATPase samples. Including the temperature correction factors of Kemper and Haigler (1982), our conversion factor systematically gives 20% lower molecular masses than those calculated by the usually employed empirical relationship published by Kepner and Macey (1968). Second, we have included an internal standard, glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides (McIntyre and Churchill, 1988), and used the decay constant of its activity as a control of interexperiment variation in the peak-to-peak peptide detection and the RIS values for disappearance of native ADP and vanadate binding sites and for inactivation of K-pNPase activity were all found to be equal to the molecular mass of one $a$-chain, $m(a)$. The RIS value for total nucleotide binding capacity and total phosphorylation was about 0.65 $m(a)$, and for Na,K-ATPase activity it approached, but did not quite reach, the double of $m(a)$. These observations will be extended and discussed in a forthcoming paper.

**EXPERIMENTAL PROCEDURES**

**Na,K-ATPase**

Na,K-ATPase was isolated in the membrane-bound form from pig kidney outer medulla by the method of Jørgensen (1974) followed by selective extraction of the plasma membranes with SDS in the presence of ATP, and isopycnic zonal centrifugation ("zonal enzyme"). The enzymes were stored at -20°C in 250 mM sucrose, 12.9 mM imidazole, and 0.625 mM EDTA at pH (38°C) = 7.15. The specific, ouabain-inhibitable activities for the two types of preparation were all purchased from Sigma. Bovine serum albumin from Behring-Merieux.

**Molecular Weight Standards**

Bacteriorhodopsin from Halobacterium, monomeric $M_c = 26,500$ (Stoeckenius and Bogomolni, 1982); glucose-6-phosphate dehydrogenase from L. mesenteroides, specific activity 800 units (NAD): (mg protein)$^{-1}$ and 6.25 mM EDTA at pH (38°C) = 7.15. The specific, ouabain-inhibitable activities for the two types of preparation were Na,K-ATPase, 20-30 and 10-15 units- (mg protein)$^{-1}$. K-pNPase about 5 and 2 units- (mg protein)$^{-1}$. Ouabain insensitive hydrolytic activity was 1-3% of total activity. The nucleotide binding capacity, see below, was about 3 and 1.5 nmol- (mg protein)$^{-1}$, respectively. Based on peptide analysis by SDS-PAGE (see below) the $\alpha$- and $\beta$-peptide made up approximately 86% of the total protein in the best preparations (zonal enzyme) and the $\alpha$- and $\beta$-peptide was found in almost equal molar concentrations.

**Materials**

SDS, Tris, and imidazole were from Sigma, EDTA and Na-p-nitrophenyl phosphate from Merck, sucrose from British Drug House, and the Na'-salts of ADP and ATP from Boehringer Mannheim. [3H]ATP and [3H]ADP were obtained as their NAD salts from The Radiochemical Center, Amersham. The nucleotides and Na'-p-nitrophenyl phosphate were converted to their Ts salts by the purification procedure of Nørbjørg and Jensen (1971).

**Enzyme Assays**

Na,K-ATPase activity was determined at 37°C by the coupled assay described by Nørbjørg (1971, 1988) with 5 mM ATP, 4 mM MgCl$_2$, 120 mM NaCl, and 25 mM KC1, 1 mM P-enolpyruvate, 0.2 mM NADH, 10 units- ml$^{-1}$ pyruvate kinase, and 30 units- ml$^{-1}$ lactate dehydrogenase. K-pNPase activity was measured at 37°C with 10 mM $p$-nitrophenyl phosphate, 20 mM MgCl$_2$, and 150 mM KC1 (Skou, 1974). Glucose-6-phosphate dehydrogenase was assayed at 37°C in 50 mM Tris-HCl, pH = 7.8 (25°C), 3 mM MgCl$_2$, 3 mM NAD, and 3.3 mM glucose-6-phosphate as described by Olive and Levy (1975).

It should be noted, that the irradiated samples were kept in the frozen state until the activities were measured. Na,K-ATPase and especially glucose-6-phosphate dehydrogenase activity were found to be sensitive to thawing and freezing, and these activities curves were therefore always measured immediately after the thawing of the samples. Likewise it is important to keep the samples under N$_2$ throughout the manipulations prior to the activity or protein-integrity measurements.

**Binding Assays**

**Nucleotide Binding Assays**—Nucleotide binding was measured at 0°C, either by using the rate-dialysis procedure as described by Nørbjørg and Jensen (1971, 1988) or by a centrifugation assay (Nørbjørg and Jensen, 1988). The medium contained 200 mM sucrose, 10 mM EDTA, 30 mM Tris, 30 mM NaCl (but no K'), and the appropriate concentrations of ATP, ADP, and [3H]ATP or [3H]ADP.

**Vanadate Binding Assays**—Vanadate binding to Na,K-ATPase was measured by a centrifugation assay (Nørbjørg and Jensen, 1988) after incubation of the enzyme with 5 mM MgCl$_2$, 10 mM KCl, 40 mM Tris (pH 7.3), and appropriate [3H]vanadate concentrations as described earlier (Hansen et al., 1979).

**Analysis of Binding Data**—The nucleotide binding isotherms with the nonirradiated Na,K-ATPase samples gave straight lines in the Scatchard plot (Jensen et al., 1984) so that the binding capacity and the dissociation constant were easily obtained. Results from the irradiated samples showed upward curved isotherms, indicative of inhomogeneity as regards the irradiation damage. These curves were resolved by assuming that there were two classes of binding sites in the irradiated samples: one class having the properties of that in the nonirradiated samples and one with decreased affinity due to irradiation. This "two-class" analysis (Jensen et al., 1984) allowed the determination of the binding capacities and, in the case of ADP binding, also the dissociation constants for the presumed two types of binding sites present in the irradiated samples.

In the vanadate binding experiments, some unspecified binding (assumed to have a site concentration and dissociation constant much larger (→) than the specific binding) was present. Correction for this yielded the capacity of sites with unchanged affinity.

**Irradiation Procedure**

The samples to be irradiated were in a medium with 250 mM sucrose, 12.9 mM imidazole, and 0.625 mM EDTA at pH = 7.15 (measured at 37°C). They were kept in perspex tubes under N$_2$ from their preparation and until analysis, and they were transported to and from the radiation facility on dry ice. The irradiations were carried out at the Accelerator Department, Risø National Laboratory, Roskilde, Denmark, normally at -10°C to -15°C. The radiation source was a linear accelerator producing 10-MeV electrons, and successive pases through the electron beam, each lasting about 3 min and exposing the samples to about 2 Mrad, were performed until the appropriate dose was reached. This procedure allowed intermitent control of sample temperature. The dose was measured by calorimetry for each pass with an accuracy well within ±5% (Miller, 1984). The methodological controls, the analysis of the samples, and the data analysis are described under "Experimental Procedures" and "Results."

**SDS-Polyacrylamide Gel Electrophoresis**

SDS-PAGE was performed essentially as described by Laemmli (1970). 200-250 µl of the sample was mixed with 25 µl of 30% SDS and 10% mercaptoethanol, 15 µl of bromphenol blue, and 45 µl of electroforetic gel solution. Samples of 30-100 µl of this mixture were then subjected to SDS-PAGE in 5-15% gradient slab gels. The gels were stained and destained in a mixture of 40% methanol and 10% acetic acid with and without 0.25% Coomassie Brilliant Blue R-250, respectively.
Quantitative gel scanning, providing both "peak height" and "integration factor" (proportional to the area of the peak in the "scannogram") of the various bands, was performed with a LKB 2202 Ultrascan Laser Densitometer. The parameters obtained were normalized to a common lane width measured on the gel whenever necessary. Control experiments illustrating the accuracy of this procedure are described under "Results."

**Protein Determination**

Protein concentrations were measured by the method of Lowry et al. (1951) after precipitation of the sample with trichloroacetic acid. Bovine serum albumin (Behringwerke) was used as standard protein, its concentration being determined using an absorption (279 nm, 1 cm) of 0.067 for 1 g liter⁻¹.

**Analysis of Radiation Inactivation Data**

It was assumed that the property investigated, be it peptide integrity, binding site concentration, or catalytic activity, declined in a monoexponential way as a function of radiation dose, $D$ Mrad:

$$P = P_0 e^{-\gamma D}$$

or

$$A = A_0 e^{-\gamma D}$$

where $P$ and $A$ are the quantified properties (surviving intact peptide and biological activity, respectively), and subscript 0 refers to nonirradiated samples (e.g. Harmon et al., 1985; Beauregard et al., 1987). The decay constant, $\gamma$ Mrad⁻¹, is determined by linear regression of $\ln P$ or $\ln A$ versus $D$ including $P_0$ and $A_0$ in the regression analysis. Note that $\gamma$ is the reciprocal of the commonly used parameter $D_{37}$, the dose that reduces $P$ or $A$ to 37% of the zero dose value: $\gamma = 1 / D_{37}$.

**RESULTS**

**Determination of Amount of Protein on the SDS-PAGE Gels**—The target size of a particular protein and the accuracy of its determination may be dependent on the method used. Here we have used SDS-PAGE for separation of the protein components of the samples ("Experimental Procedures," and Fig. 1). The relative amount of protein in the band of interest was determined by scanning such gels. This procedure was of course only applied on the highest molecular weight band (the others are masked by degradation products of this protein), and it provides two parameters: the peak height, which is the highest density of Coomassie Blue in the band, and the integration factor, which should be proportional to the accumulated (integrated) density of that band. When these parameters are normalized to a common band width in the gels, they could both be used as a relative measure of the protein content of the band.

One way of visualizing this is given in Fig. 2 which shows for the $\alpha$-peptide band of Na,K-ATPase that essentially the same dependence of protein integrity on radiation dose is obtained whether one uses peak height or integration factor as a measurement of protein (SDS gel shown on Fig. 1). Fig. 3 shows the relationship between normalized peak height or normalized integration factor and the amount of (nonirradiated) bovine serum albumin monomer applied to a gel. Both parameters seem to be proportional to the protein amount to an extent that merits their use for determination of the amount localized in the band in question. In the following we have used normalized peak height as the parameter in target size estimation.

**The Effect of Sucrose Concentration on the Decay Constant, i.e. on Target and Radiation Inactivation Size**—In the preparation of membrane-bound Na,K-ATPase, sucrose (or glycerol) is routinely added as a cryoprotective reagent in a concentration of 0.25 M. Varying the sucrose concentration between 0.25 and 1 M is without influence on the sensitivity of albumin, K-pNPPase, and Na,K-ATPase activity to radiation (not shown). It should be noted, however, that if sucrose is omitted from the buffer, the decay constants for G-6-P dehydrogenase, Ca-ATPase activities, and albumin are significantly larger than those determined in the presence of 0.25 M sucrose. Likewise, Kepner and Macey (1968) noted that a mitochondrial ATPase preparation with 30 or 50 mM had a $D_{37}$ that was twice the $D_{37}$ of a preparation with 9 mM sucrose during freeze-drying and irradiation. McIntyre and Churchill (1955), who studied the radiation inactivation of G-6-P dehydrogenase in a number of conditions, observe a slightly higher decay constant (on the average 20%) for G-6-P dehydrogenase activity in the absence of sucrose than in the presence of 0.25–0.3 M sucrose. Since the difference is within the experimental error, they concluded that the absence or

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2. P. J. Andersen, personal communication.
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Fig. 3. Quantitative determination (relative) of protein on SDS-PAGE of albumin monomer. Different aliquots of a 0.5 mg/ml albumin monomer solution in buffer were applied to gels, subjected to electrophoresis, colored, and scanned as described. 100% corresponds to approximately 50 μg of protein.

Presence of this cryoprotectant did not affect the radiation inactivation size.

Decay Constants for the Standard Proteins and the α-Peptide of Na,K-ATPase—Four standard proteins of known molecular weight were used in the present investigation, and Fig. 4 shows the effect of irradiation on the protein integrity (determined by SDS-PAGE) for these standard proteins and for the α-peptide of Na,K-ATPase. The decay constants derived from such experiments, assuming that the dose dependence is monoeponential, are used to construct the standard curve in Fig. 5, where the decay constant is plotted as a function of the known, monomeric molecular weight of the four standard proteins. From Fig. 5 it appears that the decay constant γ is proportional to the monomeric molecular mass in accordance with the basic assumptions of the radiation inactivation method. The relation obtained is: molecular mass (Da) = 6.75 \times 10^6 (Da/Mrad) \cdot \gamma (\text{Mrad}^{-1}) or molecular mass (Da) = 6.75 \times 10^9/D_{25} (\text{Mrad}). It is apparent that the target size of the α-peptide estimated from this standard curve is about 113 kDa, which is very close to the molecular mass of 112 kDa calculated from the amino acid sequence (Shull et al., 1985).

Effect of Temperature and the Protein Composition on the Decay Constant—It has been demonstrated repeatedly, both with lyophilized and frozen samples, that the radiation sensitivity of proteins (biological functions) is temperature-dependent, but the mechanism for this must be said to be largely unknown (see below). Fig. 6 shows that when the temperature is increased by 40°C from about -55°C to about -15°C, the decay constant is increased by a factor of 1.85 \pm 0.09 (15°C); 2.14 \pm 0.10 (-55°C).

The change in \( \gamma \) (or \( D_{25} \)) with temperature is comparable to that used or reported in other studies for other enzyme activities. Fluke (1987) has discussed the radiation sensitivity

FIG. 4. Radiation-mediated breakdown of four standard proteins and the α-peptide of Na,K-ATPase. All samples were in standard buffer. They were irradiated and the peptides quantified by SDS-PAGE and scanning as described under “Experimental Procedures,” see also Fig. 3. Each curve represents one irradiation experiment, and the points are the average from two to three SDS-PAGE gels. The “α-peptide” curve (•) is obtained from Na,K-ATPase samples (zonal enzyme) with or without added albumin and glucose-6-P dehydrogenase (G-6-PDH). The curve for β-galactosidase is symbolized with (○). The lines are determined by linear regression of lnP versus dose. The intercept of this line determines the value of \( P_0 \) used; and the slope, the decay constant γ, is used to construct the standard curve in Fig. 5.

FIG. 5. Standard curve for determination of target size and radiation inactivation size from the decay constant. The line is calculated by linear regression assuming proportionality between decay constant, γ Mrad\(^{-1}\), and molecular mass. The γ-values used are: bacterial rhodopsin, 0.0339 \pm 0.0023; glucose-6-P dehydrogenase (G-6-PDH), 0.0743 \pm 0.0045; albumin monomer, 0.1035 \pm 0.0042; β-galactosidase, 0.1737 \pm 0.0033. The slope is (1.48 \pm 0.04) \times 10^{-12} \text{rad}^{-1} \cdot \text{kDa}. The horizontal, hatched bar indicates the range of determinations of decay constant for the α-peptide of Na,K-ATPase. This allows the estimation of the target size for the α-peptide to 110-117 kDa (vertical, hatched bar).


Target and Radiation Inactivation Size of Na,K-ATPase

FIG. 6. Radiation sensitivity of albumin monomer and the α-peptide of Na,K-ATPase at -15 and -55°C. Albumin monomer (1 mg ml⁻¹) or Na,K-ATPase (zonal enzyme, 1 mg of protein-ml⁻¹) in the standard buffer were irradiated and the amount of intact albumin monomer or α-peptide determined after SDS-PAGE as described under “Experimental Procedures.” The decay constants (Mrad⁻¹) corresponding to the lines are: albumin, 0.064 ± 0.002 (-55°C), 0.086 ± 0.002 (-15°C); α-Peptide, 0.138 ± 0.004 (-55°C), 0.179 ± 0.008 (-15°C).

dependence upon temperature and finds that the average factor per 190°C temperature change is 1.8 ± 0.4. In comparison the works of Kempner and Schlegel (1979), Kempner and Haigler (1982), and Kempner and Miller (1983) give factors of about 2.1, 1.91, and 1.87/100°C (see Fluke, 1987, Table II). Taken together, these values correspond to the factors of 1.3–1.5/40°C found here.

The lack of effect of the admixture of proteins or membrane fragments on the decay constant for enzymatic activities of G-6-P dehydrogenase, K-pNPase, and Na,K-ATPase is illustrated by Fig. 7. It is clear that the radiation sensitivity of G-6-P dehydrogenase activity is not influenced by the presence of highly purified, membrane-bound Na,K-ATPase (zonal enzyme) or by a less purified preparation containing various other membrane proteins (enzyme II). Likewise, the decay constants for K-pNPase as well as for Na,K-ATPase activity are independent of the purity of the preparation irradiated. It might be noted that the decay constants for all three activities in this particular experiment are about 10% lower than those found in the other experiments in this study. The reason for this is probably that especially careful cooling of the samples during the radiation experiment ensured a temperature close to -20°C in the samples. In the other experiments the temperature was generally between -15 and -10°C, and a difference of 10°C corresponds to a difference in 8–12% in decay constant in our hands (see above, and Fig. 6). It appears that the internal standard G-6-P dehydrogenase also is a precise indicator of even small temperature changes.

Radiation Inactivation Size of the Ligand Binding Function and the Partial and Full Activities of Na,K-ATPase—To obtain information on the size of the “functional unit” of Na,K-ATPase, if possible, we have measured the radiation inactivation of a number of enzymatic properties.

Fig. 8 characterizes the ADP-binding properties of irradiated and nonirradiated Na,K-ATPase. Whereas the isotherms for the native Na,K-ATPase are compatible with a homogeneous population of sites, the Scatchard plot for the irradiated samples suggests inhomogeneity of (or negative cooperativity among) the binding sites. It is logical to assume that the irradiated samples contain unmodified Na,K-ATPase molecules, with the “original,” native affinity for ADP, as well as modified molecules with a lower affinity (higher dissociation constant). This two-class analysis gives an excellent fit to the data points (Fig. 8) and yields the ADP-binding site data used in Fig. 9.

Similar experiments (not shown) were performed with ATP. The affinity for ATP is about five times higher than for ADP for the native enzyme and the change in affinity by irradiation was less (but significant) than for ADP binding. For these reasons the two class simulation of the binding isotherms could only yield the total ATP-binding capacity with sufficient accuracy (Fig. 9). For vanadate binding the presence of unspecific sites allowed the determination of sites with original affinity only (see “Experimental Procedures”).

The radiation inactivation curves for Vmax of the K-pNPase activity and the Na,K-ATPase activity are also given in Fig. 9. The decay constants (Mrad⁻¹) clearly fall in three distinct groups: total nucleotide sites (irrespective of affinity), 0.156; K-pNPase and binding capacity of unmodified sites, 0.158; and Na,K-ATPase activity, 0.286.

Table I presents a compilation of decay constants, γ, for the different properties of Na,K-ATPase and for G-6-P dehydrogenase activity. The data are obtained over the last 3 years and the corresponding target size or radiation inacti-
Target and Radiation Inactivation Size of Na,K-ATPase

![Fig. 8. Scatchard plot of ADP binding data for Na,K-ATPase (enzyme II) exposed to radiation doses of 0–16 Mrad. Na,K-ATPase (enzyme II), about 8 mg of protein ml⁻¹, was irradiated in two experiments and the ADP binding assayed (○) as described under “Experimental Procedures.” The lines are computed with the assumption that the unmodified and a modified sample contain two independent classes of binding sites in varying proportion (the 0 Mrad curves contain only one class of sites). It is furthermore assumed that the affinity for ADP of each of the two classes is independent of radiation dose (corresponding to the idea of an unmodified and a modified population of sites). This two-class analysis (Jensen et al., 1984) provides the two dissociation constants 0.362 ± 0.004 and 2.57 ± 0.10 μM, as well as the concentration of the two classes of sites (see Fig. 9).

Target size for the α-peptide and the RIS for “unchanged” (native) nucleotide sites, vanadate sites, and K-pNPPase activity are all the same as the molecular mass of the α-peptide, 112 kDa (Shull et al., 1985).

Relative to the molecular mass of the α-peptide the, RIS for total binding capacity of nucleotides is clearly smaller (0.65 times) and that for Na,K-ATPase activity (assayed under optimal conditions) is larger by a factor of 1.8. In preliminary experiments not shown here, the RIS for phosphorylation capacity was found to be equal to the RIS for total nucleotide-binding capacity suggesting that binding sites

![Fig. 9. Radiation inactivation of vanadate and nucleotide binding, K-pNPPase, and Na,K-ATPase activity of membrane-bound pig kidney Na,K-ATPase. The values for vanadate binding (×), K-pNPPase (○), and Na,K-ATPase activity (●) are all from the same irradiation experiment where samples containing Na,K-ATPase (zonal enzyme), 0.5 mg ml⁻¹ were irradiated as described. The data on ADP binding (Δ, Δ) are from the two irradiation experiments described in Fig. 8, and the total ATP binding sites (▼) were obtained in a similar experiment (not shown, see “Experimental Procedures”). The decay constants (Mrad⁻¹) corresponding to the lines calculated for the three groups of data are: Δ, △, 0.158 ± 0.002; ×, ○, Δ, 0.158 ± 0.002; ●, 0.286 ± 0.001.

### TABLE I

<table>
<thead>
<tr>
<th>Property</th>
<th>n</th>
<th>Decay constant (mean ± S.E.)</th>
<th>Target size of radiation inactivation size (mean ± S.E.)</th>
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</thead>
<tbody>
<tr>
<td>α-Peptide</td>
<td>3</td>
<td>0.170 ± 0.003</td>
<td>115 ± 4</td>
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<tr>
<td>Nucleotide sites with original</td>
<td>3</td>
<td>0.160 ± 0.003</td>
<td>108 ± 4</td>
</tr>
<tr>
<td>affinity</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Total nucleotide binding</td>
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<td>0.106 ± 0.002</td>
<td>72 ± 3</td>
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<tr>
<td>capacity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vanadate sites with original</td>
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<td>0.161 ± 0.003</td>
<td>109 ± 4</td>
</tr>
<tr>
<td>affinity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-pNPPase</td>
<td>4</td>
<td>0.157 ± 0.002</td>
<td>106 ± 4</td>
</tr>
<tr>
<td>Na,K-ATPase</td>
<td>4</td>
<td>0.280 ± 0.004</td>
<td>189 ± 7</td>
</tr>
<tr>
<td>Glucose-6-P-dehydrogenase</td>
<td>2</td>
<td>0.163 ± 0.005</td>
<td>110 ± 5</td>
</tr>
</tbody>
</table>

n is the number of independent radiation experiments. In each radiation inactivation experiment, the α-peptide was determined on one to three sets of samples, and the enzyme activity was determined on two to three (K-pNPPase, Na,K-ATPase) and three (glucose-6-P-dehydrogenase) sets of samples.

with changed affinity for ATP could also be catalytically active. Furthermore, we would like to point out that when we assay the Na,K-ATPase activity with suboptimal ATP concentrations down to 5–10 μM, we can confirm the observation of Cavieres (1987) that the RIS is decreased when the ATP concentration in the assay is decreased. These matters are
under investigation and the results will be reported and discussed in a forthcoming paper.

**DISCUSSION**

The ultimate purpose of radiation inactivation studies is to determine molecular mass equivalents to the properties tested. The empirical and theoretical basis for this has been extensively described and discussed in several reviews (Kempner and Schlegel, 1979; Jung, 1984; Harmon et al., 1985; Beauregard et al., 1987; Pollard, 1987; Vaidyathanathan and Jung, 1987). It appears that the results reported in the present paper, both with regard to protein integrity (P) and enzymatic function (A) are compatible with the straightforward statistical considerations that require the properties investigated to decrease monotonically with dose. Having determined the exponential factor $\gamma = 1/D_m$ (see "Experimental Procedures"), the problem rests in establishing the appropriate relationship between molecular mass, m, and $\gamma$.

The Standard Curve for Conversion of $\gamma$ to m—The standard curve in Fig. 5 corresponds to the following relation valid for frozen samples irradiated at $t = -10$ to $-15^\circ$C.

$$m (Da) = 6.75.10^2 (20.20) \cdot \gamma (Mrad^{-1})$$

(1)

To compare it with the frequently used relationship developed empirically by Kepner and Macey (1968):

$$m (Da) = 6.4.10^{-7} \cdot \gamma (Mrad^{-1})$$

(2)

for lyophilized preparations at room temperature, a temperature correction factor of 1.3 (Kempner and Haigler, 1982) is necessary in Equation 2, which becomes:

$$m = 6.4.10^{2.3} \cdot \gamma = 8.32.10^{1.1} \cdot \gamma$$

(3)

Thus our standard curve gives values for m that are 20% lower than those obtained by using the conventional Kepner and Macey equation (Equations 2 and 3).

The use of a standard curve (and or internal standard enzyme) in this study seems especially appropriate since Kepner and Macey (1968) have no data in the m-range between $10^5$ and $10^6$ Da. Furthermore, it should be noted that the "radiation ionization energy" calculated for our standard curve at room temperature is 54 eV (see Kepner and Macey, 1968; and Pollard, 1987, for radiation statistics). Although 20% lower than the average value of 66 eV found by Kepner and Macey (1968), it is really quite compatible with the data they employ (see their Fig. 1) in determining Equation 2.

Our standard curve (Fig. 5) is based on the assumption that the integrity of the three enzymes and the albumin monomer is destroyed with a target size corresponding to their monomers. Under these circumstances, the target size of the $\alpha$-peptide of Na,K-ATPase is equal to m of the $\alpha$-peptide monomer, and we could now use the $\alpha$-peptide as an internal standard for other functions of the Na,K-ATPase. We have also used G-6-P dehydrogenase activity as an internal standard and found (Table I) the RIS value to be very close to the molecular mass of a dimer (104 kDa), as expected by all others in this field (e.g. Chamberlain et al., 1983; McIntyre and Churchill, 1985). One might conclude that the standard curve in Fig. 5 is representative for both target size and RIS values.

**Target Size of the $\alpha$-Peptide**—This is the property that has the fewest degrees of freedom as regards interpretation. The value obtained is equal to m of the peptide. An obvious conclusion is that even if there might be close subunit contact between $\alpha$- and $\beta$-peptide or between two or more $\alpha$ peptides, this contact does not allow the transmittance of "destructive energy" from radiation. At this point we must restrict ourselves to destructions large enough to let the peptides move further in the SDS-PAGE than the $\alpha$-band, but see below.

Karlsh and Kempner (1984) obtained a median target size for the $\alpha$-peptide of $128 \pm 6$ kDa, but this is actually an average between two estimates without background correction from the gel (110 $\pm$ 5, 124 $\pm 7$ kDa) and two with (137 $\pm$ 8 and 144 $\pm 8$ kDa). Our value is 115 $\pm$ 4 kDa. If we are correct in estimating that the Kepner and Macey Equation 2 gives values that are systematically about 20% too high, then the target size with background correction in Karlsh and Kempner's paper (1984) is 141/1.2 = 118 kDa. Considering the large temperature factor, this agrees well with ours and supports the notion that the $\alpha$-peptide is destroyed with a target size corresponding to m of the $\alpha$-peptide, 112 kDa.

In our investigation reported here, we have repeatedly over the last 3-4 years found an $\alpha$-peptide decay constant of about 0.170 Mrad$^{-1}$ (Table I) and this is about 25% lower than that published by Jensen and Otolenghi (1985) for Na,K-ATPase irradiated under the same circumstances (0.21 Mrad$^{-1}$). One plausible cause for the discrepancy may be that the latter value was obtained a relatively long time after the irradiation had taken place. As mentioned, irradiated samples may be somewhat unstable and slow degradation occurs during storage, but the exact reason for the deviation is unknown. The combination of a 25% higher $\alpha$-peptide decay constant, a 10-15% lower $\gamma$-value for albumin (see Fig. 4, this paper, and Fig. 2, Jensen and Otolenghi, 1985), and the assumption of a 20% lower molecular mass for the $\alpha$-peptide than that now accepted (92 versus 112 kDa) led to the conclusion that target size corresponded to $\alpha_0$. The present study as well as that of Karlsh and Kempner (1984) does not support this. On the contrary, as discussed above, we find substantial evidence that target size is equal to the molecular mass of an $\alpha$ monomer.

**RIS of Nucleotide and Vanadate Binding**—ATP and ADP bind with high affinity to the Na$^+$-form of the $\alpha$-peptide (Nerby, 1983; Jensen et al., 1984). Vanadate, on the other hand, binds with high affinity to the K$^+$-form of the $\alpha$-peptide (Nerby, 1983). There is good reason to believe that all three ligands bind to the substrate site and that there is only one such site, albeit involving several parts of the cytoplasmic peptide loop, per $\alpha$-peptide (Nerby, 1987; Jørgensen, 1988). The binding capacity for sites with the same, original affinity as in the nonirradiated samples has a RIS equal to the molecular mass of the monomeric $\alpha$-peptide (Table I). This implies that non-hit $\alpha$-peptides have retained not only their primary structure intact but also their secondary, tertiary, and quaternary structural integrity to an extent that the substrate site can change between the two conformations $E_1$ and $E_2$ just like the native peptide. Destruction of the backbone in an $\alpha$-subunit's neighboring subunits (if there are any) is apparently without influence on its substrate site.

These observations cast doubt on the validity of Karlsh and Kempner's (1984) suggestion that the "ATP-dependent functions are sensitive to the inactness of $\beta$-chains," and we have no indication that the ATP binding of an intact, non-hit $\alpha$-chain should be in any way influenced by its contact with a $\beta$-chain.

Due to irradiation, a new type of site with somewhat lower affinity for nucleotides appears (Fig. 8). This site may be located on a fragment, with a somewhat changed substrate site, from the $\alpha$-peptides whose backbones are broken. According to preliminary experiments (not shown) the site can be phosphorylated. The RIS for nucleotide binding including this site and for total phosphorylation is about 0.65 m(\(^2\)). In a forthcoming paper these phenomena will be explored and discussed.
RIS of K-pNPPase and Na,K-ATPase—First, we would like to point out that the γ-values for these two activities are equal to those found by Jensen and Ottolenghi (1985) under similar circumstances. The RIS for K-pNPPase is equal to that of the nucleotide and vanadate-binding capacity (with unchanged affinity), to the target size of the α-peptide, and thus equal to the molecular mass of an α-monomer. In agreement with the observations of Karlsh and Kempner (1984) we are tempted to conclude that this activity is "...dependent on the intactness only of the individual α-chains and are not affected by destruction of β-chains..." or destruction of possible neighboring α-chains.

The Na,K-ATPase activity, measured at optimal ligand concentrations (e.g. 3 mM ATP) has a RIS of 189 ± 7 kDa. This is 1.65–1.8 (±0.15) times that for the α-peptide (115 kDa), the unchanged binding sites (109 kDa), and the K-pNPPase activity (106 kDa). Since it has been demonstrated by Cavieres (1987) and confirmed in our laboratory that the RIS of the Na,K-ATPase activity varies with the ATP concentration in the assay medium we shall at the moment abstain from drawing any conclusions regarding the "minimal functional unit" (Beaueregard et al., 1987) for Na,K-ATPase activity. Several previous studies, in which the RIS for Na,K-ATPase was found to be considerably higher than in this paper, have been taken as support for an (αβ2) organization in the intact but lyophilized membrane (Ottolenghi et al., 1983; Ottolenghi and Ellory, 1983; Schrijen et al., 1983; Hah et al., 1985). By contrast, the data obtained by Karlsh and Kempner (1984) and by us for the RIS of Na,K-ATPase could rather be taken as evidence against such an arrangement since for example our value of 189 ± 7 kDa is definitely significantly smaller than m for an (αβ2) oligomeric complex, 294 kDa.

The problems concerning the RIS of Na-ATPase and Na,K-ATPase activity as functions of the ATP concentration are presently under investigation and will be presented and discussed in a forthcoming paper.

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