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Radiation inactivation analysis of sarcoplasmic reticulum Ca-ATPase in membrane-bound form and in detergent-solubilized monomeric states

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The sarcoplasmic reticulum Ca-ATPase was subjected to target size analysis by radiation inactivation in various buffer conditions and after solubilization in monomeric form in non-ionic detergent and in SDS. The target size was also determined for Ca-ATPase in bidimensional crystals formed in the presence of decavanadate or lanthanide. The standardization obtained with defined monomers of Ca-ATPase shows that the target size of Ca-ATPase in the functional membrane-bound state may be ascribed to a single peptide chain, possibly with surrounding lipid. Further analysis of the radiation inactivation sizes of various partial reactions of the pump cycle, including phosphorylation and Ca^{2+} occlusion, indicated much smaller values than the target size pertaining to decomposition of the whole peptide chain. This is consistent with the existence of separate functional domains within a single peptide chain.

Radiation inactivation; Target size; Ca-ATPase; Ca^{2+} occlusion; Detergent; Membrane crystal

1. INTRODUCTION

Understanding active transport of Ca^{2+} by the SR Ca-ATPase requires definition of the minimum size of the transport unit. Results obtained with detergent-solubilized Ca-ATPase in a defined monomeric state suggest that the peptide chains of M_r 110000 independently are capable of hydrolyzing ATP and of undergoing the conformational changes associated with transport [1–4]. On the other hand, a direct test of the transport function cannot be performed with the soluble enzyme due to the uncompartmentalized nature of detergent micelles, and there is evidence from structural studies that Ca-ATPase peptides may form small oligomers in the native membrane [5–7]. A critical question is whether these oligomers are of a specific type reflecting a role of peptide–peptide interaction in the transport reaction, or they represent random clustering of the peptides in the tightly packed SR membrane.

In principle, target size analysis by radiation inactivation may provide information relating to the molecular size of proteins, independently of whether they are membrane bound or soluble [8,9]. In situ radiation inactivation experiments with Ca-ATPase in membrane-bound form have shown that the ATPase activity as well as the Ca-ATPase peptide observed on SDS gels decay monoexponentially as a function of radiation dose [10,11]. Using classical target theory and the empirically established relationship $M_r = 6.4 \times 10^5$ Mrad$/D_{37}$ [12], a molecular size of 210000–250000 was calculated [10,11]. If the target size reflects the association state of the Ca-ATPase peptides in the membrane, these results would point to a well-defined dimer. However,

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Abbreviations: SR, sarcoplasmic reticulum; Ca-ATPase, calcium plus magnesium activated adenosine-5′-triphosphatase (EC 3.6.1.38); G-6-P-D, glucose-6-phosphate dehydrogenase; D_{37}, irradiation dose at which the measured parameter has decayed to 37% of a non-irradiated control value; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; $C_{12}E_8$, octaethylene glucol monododecyl ether; CrATP, β,γ-bidentate Cr(III) complex of ATP

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there are alternative explanations of the large target size, such as inclusion of lipid in the target, or a greater radiation sensitivity of Ca-ATPase relative to that of the proteins used to derive the empirical calibration factor between radiation dose and molecular size. The chemical reactions involved in functional inactivation and in decomposition of the irradiated peptide are poorly defined and interpretation of radiation inactivation data must therefore be made with caution.

In order to obtain more insight into the molecular basis for the target size of Ca-ATPase, we have undertaken a radiation inactivation study of Ca-ATPase under various conditions. One of the critical experiments which have been performed in the present investigation is to subject Ca-ATPase peptides which are in a defined monomeric state to target size analysis. This provides a standardization which is independent of comparison of Ca-ATPase with other proteins. In addition we have determined the radiation inactivation sizes for some of the partial reactions of the pump cycle, phosphorylation and Ca^{2+} occlusion. Our results clearly demonstrate that an oligomeric state of the Ca-ATPase protein in the membrane cannot be inferred from radiation inactivation, and that classical target theory ('one hit inactivation') is inapplicable to this system. However, useful information concerning the relative size of various functional domains of Ca-ATPase can possibly be obtained by the target size analysis.

2. MATERIALS AND METHODS

SR vesicles and purified Ca-ATPase membrane were prepared from rabbit skeletal muscle as described [13]. For irradiation, the membranes were suspended at a concentration of 0.3–1 mg protein/ml in buffer containing 20 mM Tes (pH 7.0), 130 mM NaCl, together with sucrose and other additions as described in the figure legends. When present, benzoic acid (10 mM) was added from a 100 mM stock solution adjusted to pH 7.0.

Soluble Ca-ATPase preparations in a predominantly monomeric form [1,13] were prepared by addition of 10 mg $C_{12}E_8$/ml or 10 mg SDS/ml to 0.3 mg purified Ca-ATPase/ml in 20 mM Tes (pH 7.0), 130 mM NaCl, 0.5 mM $CaCl_2$, 0.5 M sucrose.

Membrane crystals [16] were formed by overnight incubation of SR (1 mg protein/ml) in the presence of either 5 mM $NaVO_4$, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM Tes (pH 7.0), 130 mM NaCl (vanadate-induced crystals) or in the presence of 25 μM $LaCl_3$, 5 mM $MgCl_2$, 20 mM Tes (pH 7.0), 130 mM NaCl (lanthanide-induced crystals). The vanadate stock solution was kept at pH 2.0 for some hours before use, to induce decavanadate formation [17]. The presence of decavanadate was indicated by a yellow colour. When present during irradiation, 0.5 M sucrose was added to the crystals immediately before freezing of the samples.

All samples were flushed with N_2 before the tubes were sealed and frozen at −80°C. Irradiation was carried out at a temperature of −55 ± 5°C with 10 MeV electrons (using the linear accelerator at The Danish Atomic Energy Commission, Risø). Before thawing for assay, flushing with nitrogen was repeated. Some experiments, that are not presented in this article, were also carried out at a radiation temperature of −10 to −20°C. The results were qualitatively similar to those obtained at the lower temperature.

Irradiation doses were measured by thermocalorimetry. For data presentation, all doses were normalized to the relation $M_r = 6.4 \times 10^5$ Mrad/D_{37} [12] on the basis of results obtained in parallel experiments with the recommended standard enzyme, G-6-P-D from *Leuconostoc mesenteroides* [14]. Thus the relation used for dose calculation was the following

$$\text{Actual dose} = \frac{\text{dose measured by thermocalorimetry}}{D_{37} \text{ for G-6-P-D measured by thermocalorimetry}} \times \frac{6.4 \times 10^5 \text{ Mrad}}{104000}$$

104000 being the presumed molecular mass of the standard enzyme [14]. Apparent target sizes for Ca-ATPase were calculated from the relation

$$\text{Apparent target size} = \frac{D_{37} \text{ for G-6-P-D}}{D_{37} \text{ for Ca-ATPase}} \times 104000$$

Note that the standard enzyme was always present in the same buffer (20 mM Tes, pH 7.0, 130 mM NaCl, 0.5 M sucrose), whereas the buffer conditions for Ca-ATPase were varied on purpose.

Ca-ATPase activity was measured by the NADH-coupled assay described in [1] in the presence of 5 mM MgATP and 0.1 mM $CaCl_2$. The irradiated protein was diluted more than 100-fold in the assay medium.

Phosphorylation from [γ-^{32}P]ATP was measured essentially as in [13] in the presence of 20 mM Tes (pH 7.0), 130 mM NaCl, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$ and 50 μM [γ-^{32}P]ATP.

CrATP-induced Ca^{2+} occlusion was measured by Millipore filtration as described in [4], after 5 h incubation in the presence of 500 μM $^{45}Ca^{2+}$, 1 mM CrATP and 10 mM $MgCl_2$, 20 mM Tes (pH 7.0), 130 mM NaCl. In experiments where the occluded state was produced before irradiation, sucrose was not added until immediately before freezing of the samples.

Ca^{2+} occlusion induced by the physiological substrate, ATP, was measured by quenching with $LaCl_3$ [15]. The samples of Ca-ATPase were diluted to 0.2 mg protein/ml and phosphorylated by ATP (50 μM) at 0°C in the presence of 20 mM Tes (pH 7.0), 0.3 M KCl, 100 μM $^{45}CaCl_2$, 1.5 mM phospho*enol*pyruvate, and 0.1 mg pyruvate kinase/ml. After 15 s, 100 μl were transferred to 5 ml of quenching solution containing 20 mM Tes (pH 7.0), 0.3 M KCl, 2 mM $LaCl_3$ and filtered. The protein deposit on the filters was washed with 15 ml of the quenching solution.

SDS polyacrylamide gel electrophoresis was carried out essentially as described in [18] except that heating of the samples at 100°C was avoided. Quantitation of the amount of Ca-ATPase peptide present on the Coomassie brilliant blue stained gels was performed with an LKB 2202 Ultroscan Laser Densitometer, using the integrator function.

The standard enzyme, G-6-P-D, used for calibration of irradiation dose was assayed essentially as described [14].

3. RESULTS

Fig.1 shows the effect of sucrose (0.5 M) and of benzoic acid (10 mM) on the relationship between remaining Ca-ATPase activity and radiation dose. For these studies a purified membrane-bound Ca-ATPase preparation [13] was used. Sucrose was found to protect against inactivation, the effect being saturated above 0.25 M sucrose. When benzoic acid was added with 0.5 M sucrose, radiation sensitivity declined further. Benzoic acid is known as a free radical scavenger, and variable effects on radiation sensitivity of other proteins have been reported [19]. The effect of sucrose may also be due to radical scavenging, but apparently the maximum protection obtained with sucrose alone is less than that obtained with sucrose in combination with benzoic acid. It should be noted that due to dilution neither of these protective compounds

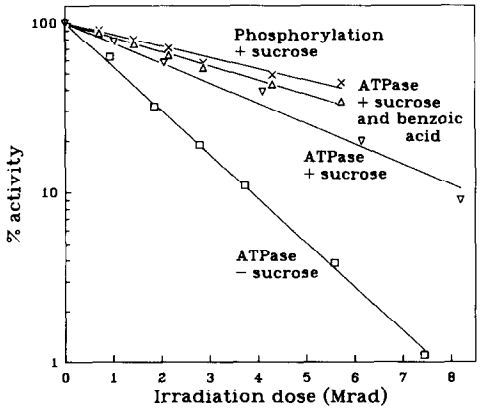

Fig.1. Radiation inactivation of Ca-ATPase. Effect of sucrose and benzoic acid. Purified Ca-ATPase membranes were irradiated in the presence of 0.5 mM Ca^{2+} with (▽, △, ×) or without (□) 0.5 M sucrose. Benzoic acid (10 mM adjusted to pH 7.0) was present in (△). ATPase activity (▽, △, □) was measured after 100-fold dilution of the samples in buffer without sucrose; 100% values were 5.3 μmol/min per mg. Phosphorylation from [γ-^{32}P]ATP (×) was measured in the sample irradiated in the presence of 0.5 mM sucrose and absence of benzoic acid, after a 10-fold dilution in the same medium as used for determination of ATPase activity; 100% values were 4.1 nmol/mg of protein.

were present in significant amounts during the ATPase assay. Therefore, their effects were exerted in the frozen state (during irradiation) or during thawing of the irradiated samples. Other variations of buffer conditions were also tested. These included substitution of Tris buffer for Tes, removal of NaCl, and variation of pH between 7.0 and 8.0. The effects were modest (<25% changes of slopes of decay curves) relative to those induced by sucrose and benzoic acid.

When the recommended standard protein G-6-P-D [14] was subjected to the same buffer variation as shown in fig.1, the changes in radiation sensitivity were qualitatively similar to those observed with Ca-ATPase, but less pronounced quantitatively. Assuming a radiation inactivation size of 104 kDa for G-6-P-D, a value of 175000 could be calculated for the radiation inactivation size of Ca-ATPase in the presence of 0.5 M sucrose and absence of benzoic acid. This is in reasonable agreement with the radiation inactivation size of 210000–250000 determined by Hymel and co-workers [10] under similar conditions, and with that of 190000 determined for a lyophilized sample [20]. The medium containing 0.5 M sucrose was used in the experiments described below.

Fig.1 also shows decay of the capacity of Ca-ATPase for Ca^{2+}-dependent phosphorylation from [γ-^{32}P]ATP in the presence of 0.5 M sucrose. The radiation inactivation size for this partial reac-

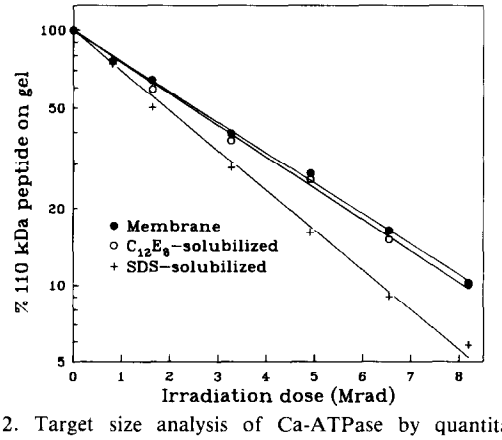

Fig.2. Target size analysis of Ca-ATPase by quantitative scanning of SDS-polyacrylamide gels. Effect of solubilization in detergent. Purified Ca-ATPase was irradiated in the presence of 0.5 M sucrose and 0.5 mM Ca^{2+} in the membranous form (•) and after solubilization in monomeric form in $C_{12}E_8$ (○) or in SDS (+) as described in section 2.

tion was considerably smaller (102000) than that pertaining to ATPase activity. This could mean that two ATPase chains are required for maximum ATP hydrolysis, but not for phosphorylation. However, the results presented below seem to contradict this interpretation.

In the experiments shown in fig.2, the decomposition of Ca-ATPase peptide as a function of radiation dose was studied by gel scanning after SDS polyacrylamide gel electrophoresis. We have confirmed the finding in [10] that the 110 kDa peptide disappears with a slope corresponding to the same target size as that obtained by analysis of Ca-ATPase activity. This large target size may be explained by dissipation of radiation damage through non-covalent bonds between the two polypeptide chains in a dimeric complex [10] or between one peptide and the surrounding lipid. In order to examine the former possibility, the radiation sensitivity of the peptide was examined after solubilization in the non-ionic detergent $C_{12}E_8$, and also after solubilization in SDS. In the unfrozen state the enzyme solubilized in excess $C_{12}E_8$ is present as defined monomers in the detergent micelles [1,2,13,21], and it is not expected that specific dimers form upon freezing. In SDS, the denaturation of the peptide chains precludes any specific peptide–peptide interactions. It is seen from fig.2 that the enzyme solubilized in $C_{12}E_8$ displays a target size similar to that of the membrane-bound Ca-ATPase, whereas the target size determined in SDS is about 30% larger. On the other hand, the target size would have been expected to decrease upon solubilization in monomeric form, if energy transfer between peptides in a non-covalent dimeric complex contributed to the target size in the membranous state.

Since it has been demonstrated that Ca-ATPase in the native SR membrane can be induced to form bidimensional crystal lattices with either a dimeric or a monomeric unit cell [16], we also examined the target sizes for decomposition of Ca-ATPase peptide in these crystals (fig.3). After incubation of SR with decavanadate in the absence of Ca^{2+} to obtain crystals with a dimeric unit cell, the target size was found to be identical to that observed in non-crystalline SR preparations in the presence of Ca^{2+}. For undefined reasons the size measured with either Ca^{2+} or vanadate present (150000) was slightly smaller than that observed in the above

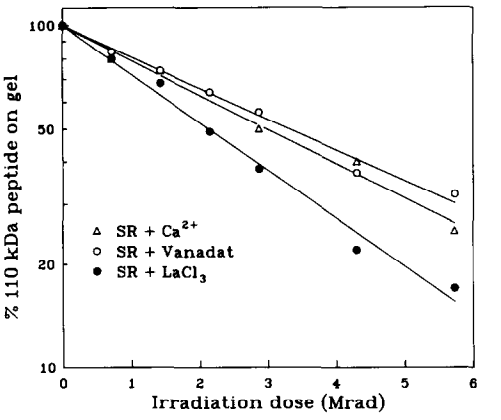

Fig.3. Target size analysis of Ca-ATPase by quantitative scanning of SDS polyacrylamide gels. Effect of formation of bidimensional crystals. Δ, SR vesicles irradiated in the presence of 0.5 mM Ca^{2+}; ○, SR vesicles irradiated in the presence of 5 mM $NaVO_4$, 5 mM $MgCl_2$, 1 mM EGTA (crystal with dimeric unit cell); ●, SR vesicles irradiated in the presence of 25 μM $LaCl_3$, 5 mM $MgCl_2$ (crystal with monomeric unit cell). Sucrose (0.5 mM added immediately before freezing) was present in all samples.

described studies of purified Ca-ATPase membrane, under otherwise similar conditions.

Incubation of SR with $LaCl_3$ to obtain crystals with a monomeric unit cell led to a target size of approximately 200000. These experiments were repeated in the absence of sucrose, again with the result that the target sizes in the presence of Ca^{2+} or decavanadate were identical, whereas the target size in the presence of $LaCl_3$ was slightly larger.

The previously described finding that the radiation inactivation size for phosphorylation was smaller than that measured for Ca-ATPase activity (fig.1) prompted us to examine other partial reactions in the pump cycle. A low-affinity modulatory effect of ATP on ATPase activity has previously been ascribed to subunit interaction [22,23], but recent evidence suggests that this effect may be accounted for by the properties of the catalytic site in various conformational states of a single peptide chain [24,25]. In the present investigation we compared the effects of irradiation on ATPase activity measured at low (10 μM) and high (5 mM) ATP concentration. In contrast to the analogous experiments carried out with the Na,K-ATPase [26], there was no difference between the decay curves obtained for the two ATP concentrations with Ca-ATPase. Therefore for Ca-ATPase, the enhance-

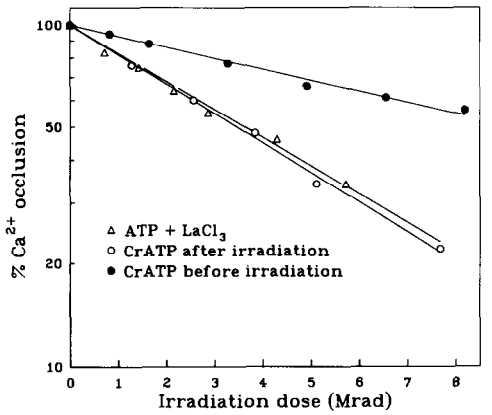

Fig.4. Radiation inactivation of Ca^{2+} occlusion. In (○, △), Ca^{2+} occlusion was examined after irradiation in the presence of 0.5 mM $^{45}Ca^{2+}$. Either CrATP (5 h incubation, cf. [4]) (○), or ATP with $LaCl_3$ quenching (cf. [15]) (△) was used to stabilize the occluded intermediate during measurement by filtration. In (●), incubation with CrATP and $^{45}Ca^{2+}$ was performed before freezing and irradiation, to measure the effect of irradiation on maintenance of the occluded state.

ment of catalytic activity at millimolar ATP concentrations does not appear to be ascribable to a larger functional unit than that involved in hydrolysis of ATP present at low concentrations.

A central event in the transport process is believed to be the occlusion of Ca^{2+} in the phosphorylated intermediate [27,28]. It was previously demonstrated that the occluded form of the enzyme could be stabilized using β,γ-bidentate CrATP as phosphorylating substrate [4,29]. It can be seen in fig.4 that the radiation inactivation size obtained for the CrATP-induced Ca^{2+} occlusion in purified membrane-bound Ca-ATPase is 125000, i.e., not significantly different from that observed for phosphoenzyme formation in fig.1. The Ca^{2+}-occluded form can also be stabilized by addition of $LaCl_3$ after phosphorylation by the physiological substrate, ATP [15]. As seen in fig.4 studies of this reaction yielded a radiation inactivation size for Ca^{2+} occlusion identical to that obtained with CrATP.

In another set of experiments the occluded state was formed before irradiation of the samples, and the effect of irradiation on maintenance of Ca^{2+} occlusion was studied. Fig.4 shows that the corresponding radiation inactivation size was smaller (approximately 45000) than that involved in the initial formation of the Ca^{2+}-occluded intermediate.

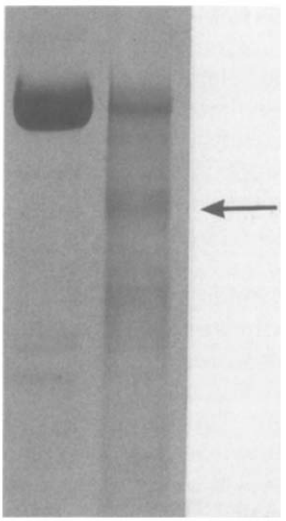

Fig.5. SDS polyacrylamide gel electrophoresis showing formation of distinct peptide fragments as a result of irradiation. Left, non-irradiated control sample. Right, Ca-ATPase subjected to 6.5 Mrad irradiation in the presence of 0.5 mM Ca^{2+} and 0.5 mM sucrose. The arrow indicates fragments of M_r 50000–70000.

The observation of radiation inactivation sizes for phosphorylation and Ca^{2+} occlusion, which are smaller than that associated with decomposition of the Ca-ATPase peptide, can be explained by formation of peptide fragments corresponding to functional domains. We therefore looked for evidence of well-defined fragments on the SDS gel patterns obtained after irradiation. It is seen in fig.5 that a distinct band is formed in the 50–70 kDa region (arrow). In the lower molecular mass regions there are multiple less distinct bands.

4. DISCUSSION

In the present investigation target size analysis was applied to samples containing Ca-ATPase peptides in well-defined self-association states, and the results were compared with data obtained with functional membrane-bound Ca-ATPase of unknown self-association state.

In one type of standard Ca-ATPase sample, detergent was added to dissociate peptide–peptide associations. Investigations of other membrane-bound and soluble proteins have indicated that effects of detergent per se on radiation sensitivity are

rather modest, at most amounting to a 30% increase of apparent target size [30,31].

In another type of standard sample, the Ca-ATPase was membrane bound, forming bidimensional crystals with either a dimeric or a monomeric unit cell.

In none of the monomeric Ca-ATPase preparations did the target size assume a value smaller than that measured in the usual (non-crystalline) membrane-bound Ca-ATPase preparations and in the crystal lattice with dimeric unit cell. Therefore, there is no firm basis for attributing the target size of Ca-ATPase to subunit interaction in a dimer.

The question then arises why the Ca-ATPase possesses such a large target size relative to that expected from the peptide molecular mass of 110 kDa. One explanation could be that the region of Ca-ATPase close to the lipid phase exposes certain amino acids (e.g. tryptophans), which are particularly potent in transmission of radiation damage from the surroundings. The effect of benzoic acid on the radiation sensitivity indicates that radical mechanisms are involved in the reactions leading to inactivation, but there is insufficient knowledge about details of the chemistry [9,19]. Interestingly, the closely related Na,K-pump protein behaves differently from Ca-ATPase in radiation inactivation studies, since the target size observed by SDS gel electrophoresis corresponds closely to the molecular mass of the 112 kDa α-subunit ([32], and Jensen, J., personal communication). This may be related to the fact that in Na,K-ATPase only 2 of a total of 12 tryptophans in the α-subunit are located in predicted transmembrane segments [33], whereas all except 2 of the 13 tryptophans in Ca-ATPase are in the presumptive membrane region [34]. There is, however, no experimental foundation of this speculation at present.

The target sizes obtained with the detergent-solubilized enzyme would be consistent with energy transfer from surrounding lipid or detergent. The amount of $C_{12}E_8$ bound per soluble peptide in g/g protein is of a magnitude similar to the amount of lipid interacting with the protein in the membranous state [21,35]. For SDS solubilized protein, which is in an unfolded state, there is more detergent bound [36], and under these conditions the target size was found to increase by about 30%.

The radiation inactivation sizes for the phosphorylation reaction and for Ca^{2+} occlusion were found to be smaller than the radiation inactivation size pertaining to ATPase activity and the target size for decomposition of the whole peptide chain. This means that the classical assumption in target theory, that one hit destroys a whole target, does not hold for Ca-ATPase. Based on the appearance of distinct bands on the gel, it may be suggested that peptide fragments corresponding to well-defined functional domains are formed upon irradiation. Recently, a model for the Ca-ATPase peptide structure was proposed, consisting of subdomains for nucleotide binding, phosphorylation, Ca^{2+} binding, and 'transduction' [34]. Relative to the target size of the whole peptide chain, the radiation inactivation sizes that we observed for ATPase activity, phosphorylation-induced Ca^{2+} occlusion, and maintenance of the Ca^{2+}-occluded state, once it had been formed, were 100, 60–70, and 26%, respectively. In the light of the uncertainties mentioned above, the fit with the structure model does not seem too bad, if it is assumed that the Ca^{2+} occlusion induced by phosphorylation from ATP or CrATP involves cooperation between the phosphorylation domain, the nucleotide-binding domain, and the Ca^{2+}-binding domain, whereas the maintenance of the occluded state requires intactness of only phosphorylation and Ca^{2+}-binding domains. From the large radiation inactivation size pertaining to ATPase activity (100% of the target size of the peptide chain) it may be surmised that the complete transport cycle requires additional intactness of the 'transduction' domain and of the whole transmembrane region. This would be in accordance with a transport model in which the movement of the Ca^{2+} through the membrane (presumably the most complex part of the ATPase reaction) occurs after phosphorylation and Ca^{2+} occlusion rather than in direct connection with these events (cf. [28,34]).

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