The Animal and Human Plasma Membrane (Ca^{2+}+ Mg^{2+})-ATPases—Approaches to Molecular Arrangements of Functional Parts and Oxidative Changes

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Abstract. The molecular structures of animal and human plasma membrane (Ca^{2+}+ Mg^{2+})-ATPases are not completely understood in part due to the fact that no suitable single crystal is available. The elucidation of the two-dimensional structure is in progress. The amino acid sequences of human erythrocyte and rat plasma membrane Ca^{2+} pump isoforms as well as of the pig smooth muscle plasma membrane Ca^{2+} pump are already known. This article reviews the present state of the knowledge in (Ca^{2+}+ Mg^{2+})-ATPase research of animal and human plasma membranes performed in the past few years, concerning in particular

- arrangements of proteolytically cleaved fragments, and relations between the erythrocyte (Ca^{2+}+ Mg^{2+})-ATPase *in situ* and the purified red cell enzyme,
- oxidative changes.

Results of different experimental approaches concerning the structure of (Ca^{2+}+ Mg^{2+})-ATPases rather than the applications of the methods used are emphasized.

Key words: (Ca^{2+}+ Mg^{2+})-ATPase — Ca^{2+} pump — Ca^{2+} transport — Plasma membrane — Oxidative changes — Superoxide anion radical (O_2^-) — Tert-Butyl hydroperoxide — Electron spin resonance (ESR) —Iron ion —Ferric (Fe^{3+}) — Ferrous (Fe^{2+}).

Abbreviations: DTT, 1,4-dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid; EP, phosphorylated intermediate of the enzyme; ESR (EPR), electron spin (paramagnetic) resonance; GSH, reduced glutathione; PAGE, polyacrylamide (slab) gel electrophoresis; PCMB, *p*-chloromercuribenzoate; SDS, sodium dodecyl sulfate.

I. Introductory remarks

Although no details of the structure of (Ca^{2+}+ Mg^{2+})-ATPases from various cell types are completely known, progress in understanding selected properties

of (Ca^{2+}+ Mg^{2+})-ATPases has been made on the basis of model investigations. Experiments in the recent years promoted a discussion with regard to:

- which enzymes are Ca^{2+} pumps,
- in which states do they exist,
- which molecular structures can be found in these states,
- how do these molecular structures arise,
- by what mechanisms do the reactions take place and how fast,
- what are the physiological functions of (Ca^{2+}+ Mg^{2+})-ATPases,
- how are these processes regulated.

Due to the lack of precise knowledge about the structure and structure-function relationships (Scarborough 1982; Carafoli 1985; Pedersen and Carafoli 1987a,b; Dhalla and Zhao 1988; Serrano 1988; see Fleischer and Fleischer 1988) a certain free choice of classification of ATPases is inevitable. The erythrocyte (Ca^{2+}+ Mg^{2+})-ATPase localized exclusively in the plasma membrane, and the (Ca^{2+}+ Mg^{2+})-ATPase of the sarcoplasmic reticulum membrane of the heart muscle have been studied most intensively and are the best characterized (Ca^{2+}+ Mg^{2+})-ATPases. Therefore, they are being used as a reference to ATPases of different sources.

General views on approaches to elucidate the structure

Molecular structures of animal and human plasma membrane (Ca^{2+}+ Mg^{2+})-ATPases are not completely understood in part due to the fact that no single crystal has so far been available. The three-dimensional structure remains unknown. The elucidation of the two-dimensional structure is in progress. The amino acid sequences of human erythrocyte (Verma et al. 1988; Strehler and Carafoli 1988; Strehler et al. 1990) and rat (Shull and Greeb 1988; Greeb and Shull 1989) plasma membrane Ca^{2+} pump isoforms as well as of the pig smooth muscle plasma membrane Ca^{2+} pump (De Jaegere et al. 1990) are already known. In other words, at present it is impossible to get a clear image or to make an acceptable generalization without phenomenological approach to the structural questions. Hence, different approaches and results thereof are used to describe the structure. Radical reactions and oxidative changes are also involved. Radical reactions cause undesirable structural changes; nevertheless, they could be used systematically to elucidate the structure. With this intention, a model was designed of the Ca^{2+} pump *in situ* (Sarkadi et al. 1978, 1980a,b, 1986a; Enyedi et al. 1980, Stieger and Schatzmann 1981).

On this background and the present state of art, the article aims at discussing some aspects of animal and human plasma membrane (Ca^{2+}+ Mg^{2+})-ATPases based on a critical review of the literature and on some own results in the field.

II. (Ca^{2+}+ Mg^{2+})-ATPases of animal and human plasma membranes: past and recent focus

Recent studies were focused on disclosing relations between:

- the molecular structures of different(Ca^{2+}+ Mg^{2+})-ATPases,
- the phosphorylation/dephosphorylation of the enzyme intermediates,
- the Ca^{2+} transport.

Most of the studies were performed on the non-purified or purified enzymes of human or rat erythrocyte membranes, human thrombocyte membranes and sarcoplasmic reticulum membranes of rabbit heart muscle at the level of proteolytically cleaved polypeptides. The molecular weight of Ca^{2+} pumps, the Ca^{2+}-dependent phosphorylation and the active Ca^{2+} transport after limited proteolysis, as well as dephosphorylation and calmodulin binding were measured. Purification and reconstitution of plasma membrane Ca^{2+} pumps (see Penniston et al. 1988), isolation and reconstitution of Ca^{2+} pumps from human and porcine platelets (see Dean 1988), and reconstitution of Ca^{2+} pumping of the cardiac sarcoplasmic reticulum (see Inui and Fleischer 1988) were described in detail.

II-A. Former ideas concerning the structure

Former proposals for the structure of (Ca^{2+}+ Mg^{2+})-ATPases reached different levels of abstractions (Scharff 1976, 1978, 1981; Scharff and Foder 1978; Gopinath and Vincenzi 1977; Jarrett and Penniston 1977; Wolf et al. 1977; Sarkadi et al. 1980b: Enyedi et al. 1980; Mauldin and Roufogalis 1980; Maretzki et al. 1980, 1981, 1982; Reimann et al. 1981; Pedemonte and Balegno 1981; Carafoli et al. 1982; Carafoli and Zurini 1982: Luterbacher and Schatzmann 1983). Therefore, attention was focused on detailed investigations of the properties (Carafoli et al. 1982; Penniston 1982; Schatzmann 1982) and relations between the properties of (Ca^{2+}+ Mg^{2+})-ATPases (Carafoli et al. 1982; Schatzmann 1982; Sarkadi et al. 1982, 1986a; Enyedi et al. 1985, 1986). Comparable properties offer a basis for relating structural and/or functional aspects of (Ca^{2+}+ Mg^{2+})-ATPases of membrane fractions of different intracellular or cellular origins or of unknown subcellular origin.

II-B. The Ca^{2+} pump *in situ*

The Ca^{2+} pump *in situ* exists in a characteristic microenvironment resulting from the effects of the membrane on which it is located. Enzymes extracted from membranes often show different properties compared with the non-purified enzymes(Racker 1967; Coleman 1973). Thus, to study the enzyme, it is better to

preserve its natural environment as far as possible. Red cell Ca^{2+} pump was prepared with special care to maintain the Ca^{2+} pump within the hydrophobic areas of the inside-out membrane vesicles (Sarkadi et al. 1980a). Complex structural and functional properties, and especially relations between these properties, of Ca^{2+} pumps *in situ* are criteria by which to characterize (Ca^{2+}+ Mg^{2+})-ATPases.

A detailed methodological work has been done to solve one of the methodological problems in studies using non-purified plasma membrane Ca^{2+} pumps: their molecule-specific detection, i.e. the so-called "visualization" (Sarkadi et al. 1986a). Visualization of non-purified plasma membrane Ca^{2+} pumps has been difficult, since the stability of phospho-acyl-intermediates strongly depends on the pH value used (Lichtner and Wolf 1980a; see Stekhoven and Bonting 1981; Heilmann et al. 1985), and since they occur in low concentrations in plasma membranes (Graf et al. 1980; Schatzmann 1982); the molecular weight and solubilization properties are similar to those of Band 3 polypeptides (Szász et al. 1978; Niggli et al. 1981b; Schatzmann 1982; Graf et al. 1982). Typical approaches to revealing of differences between (Ca^{2+}+ Mg^{2+})-ATPases (Sarkadi et al. 1986a; Enyedi et al. 1985, 1986) have been:

(i) the development of an acidic discontinuous slab SDS-PAGE system to maintain the concentrations of the several phospho-acyl-intermediate species, and at the same time to provide a good resolution of the non-purified plasma membrane pump protein;

(ii) the application of low concentrations of ATP (0.1–0.3 μmol/l, including γ^{32}P-ATP) in the absence of added Mg^{2+} and in the presence of Ca^{2+} or Ca^{2+} and La^{3+} at 4°C (that warranted exclusive phosphorylation of the non-purified Ca^{2+} pump and a stable maximum level of phosphoenzyme intermediates, even of the proteolytically activated Ca^{2+} pumps).

The experimental approaches mentioned above resulted in the development of new ideas concerning the molecular structures and the mechanism of action of the red cell (Ca^{2+}+ Mg^{2+})-ATPase *in situ*, i.e. in the most approximate natural environment. The two types of (Ca^{2+}+ Mg^{2+})-ATPases *in situ* were further characterized by defining differences between them.

II-C. Direct approaches to study the molecular structures

Up to this point no direct probes of the molecular structures of animal and human plasma membrane (Ca^{2+}+ Mg^{2+})-ATPases were available. This lack of information can be remedied by:

- functional and structural mapping of the polypeptide regions of the animal and human plasma membrane Ca^{2+} pump proteins with polyclonal and monoclonal antibodies (Sarkadi et al. 1988a,b,c; Papp et al. 1988, 1989);

- amino acid sequence analysis (Filoteo et al. 1987; James et al. 1987, 1988, 1989b; Carafoli 1988);
- cloning techniques (Carafoli 1988; Strehler and Carafoli 1988; Verma et al. 1988; Strehler et al. 1989, 1990; Shull and Greeb 1988; Greeb and Shull 1989; Brandt et al. 1988; Mann et al. 1989; Enouf et al. 1990; De Jaegere et al. 1990);
- stabilization and crystallization of the purified enzyme. Crystallization of the Ca^{2+} pump was successful in the sarcoplasmic reticulum only (Dux and Martonosi 1983a,b; Pikula et al. 1988; Taylor et al. 1988a,b; Müllner et al. 1988; Martonosi et al. 1990; Molnar et al. 1990).

The Ca^{2+} binding peptide region of skeletal (Ca^{2+}+ Mg^{2+})-ATPase and cardiac (Ca^{2+}+ Mg^{2+})-ATPase (Gangola and Shamoo 1986) as well as peptides of the calmodulin binding domain of the erythrocyte Ca^{2+} pump (Enyedi et al. 1989; Vorherr et al. 1990) have been synthesized and characterized.

III. Arrangements of proteolytically cleaved fragments and relations between the erythrocyte (Ca^{2+}+ Mg^{2+})-ATPase *in situ* and the isolated purified red cell enzyme

The number and the nature of proteolytically cleaved fragments depend, to a large extent, on the use of either the enzyme *in situ* or the purified (Ca^{2+}+ Mg^{2+})-ATPase. Based on the data obtained from experiments with limited proteolysis it was found that the cleaving sites are determined by the properties of the holoenzyme or of the partially cleaved enzyme, such as the primary amino acid sequence, the accessibility for proteases, and the micromedium (membrane environment). Selected properties of the enzyme or its cleavage products, such as phosphorylation, Ca^{2+} transport or calmodulin binding, do not affect the cleaving sites.

III-A. Proteolysis pathways

Originally suggested by Sarkadi et al. (1986a) for the red cell Ca^{2+} pump *in situ*, phosphorylation, Ca^{2+} transport and calmodulin binding are different for the fragments A to D (Fig. 1). Three "cuts" or "sections" mapping the Ca^{2+} pump molecule *in situ* in different phosphorylated and non-phosphorylated fragments were suggested. With trypsin producing subsequently "cut 1", "cut 2", and "cut 3", phosphorylated fragments of approx. molecular weights of 125 kDa, 90 kDa and 80 kDa are obtained. Chymotrypsin that produces "cut 3" and "cut 2" yields 130 kDa and 80 kDa phosphorylated fragments. Pronase and papain producing "cut 2" and "cut 3" yield 90 kDa and 80 kDa phosphorylated fragments. With carboxypeptidase A producing "cut 3" a phosphorylated fragment of approx. 130

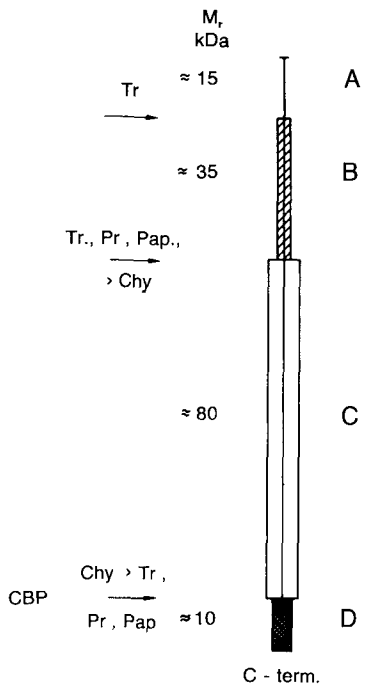


Figure 1. Putative model for the arrangement of the proteolytically cleaved functional parts in the primary structure of the erythrocyte calcium pump protein. A: 15 kDa, easily trypsin-cleavable fragment; B: 35 kDa, hydrophobic (membrane-bound?) fragment, associates with fragment C under acidic conditions in SDS; C : 80 kDa, fully active calcium pump, membrane-bound, binds and splits ATP, forms EP, binds and transports Ca^{2+}, can be inhibited by La^{3+}; D : 10–12 kDa regulatory fragment, binds calmodulin, inhibits the activity of the pump in the absence of calmodulin. Tr.= trypsin; Pr.= pronase; Pap.= papain; Chy.= chymotrypsin; CPB. = carboxypeptidase A. (Reproduced from Sarkadi et al. 1986a).

kDa is obtained. It has to be noted that proteolytic cleavings proceed under prolonged proteolysis. The appearance of a phosphorylated fragment smaller than 80 kDa was expected from an exposure to higher trypsin concentrations and on prolonging digestion times. Indeed, the experimental conditions to detect a 76 kDa phosphorylated fragment could be established (Enyedi et al. 1987; Papp et al. 1989). Identification has been based on indirect evidence, no direct procedures were available. The accessibility of all the fragments from the cytoplasmic leaflet of the erythrocyte membrane, i.e. from the outside of the red cell inside-out membrane vesicles, suggests a looped arrangement of the Ca^{2+} pump molecule within the red cell membrane. It seems reasonable to conclude that the 15 kDa fragment obtained in the easiest way by trypsin digestion, and the 10 kDa fragment, are located at the cytosolic leaflet of the erythrocyte membrane. In a recent study, it was shown that calpain-I digestion produces a 125 kDa phosphorylated fragment (Papp et al. 1989). If a 90 kDa fragment is already present, calpain-I yields an 81 kDa phosphorylated fragment. Proteolytic fragmentation and amino acid composition of the isolated and purified red cell Ca^{2+} pump were analyzed in detail in the past (Niggli et al. 1979a,b, 1981a,b; Graf et al. 1982; Carafoli et al. 1982; Carafoli and

Zurini 1982; Zurini et al. 1984; Benaim et al. 1984; Enyedi et al. 1987; James et al. 1989a). At this stage, numerous degradation products were obtained (at least 14). The number of proteolytic fragments does not correlate with functional or structural properties of the purified enzyme. More recently, the purified red cell Ca^{2+} pump has been exposed to trypsin under conditions designed to enrich the 90 kDa, 85 kDa and 76 kDa fragments (Zvaritch et al. 1990).

III-B. Functional domains

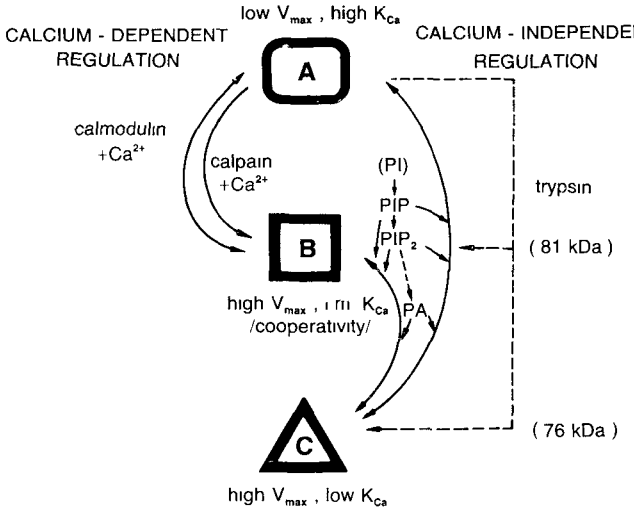
The portions of the erythrocyte Ca^{2+} pump *in situ* which bind monoclonal antibodies raised against the purified red cell Ca^{2+} pump molecule could be detected. Three groups of monoclonals were revealed after separation of the phosphoprotein on nitrocellulose, reaction with monoclonal antibodies, peroxidase staining and autoradiography of the same blot (Sarkadi et al. 1988a,b,c). Monoclonal antibodies (**A**) are directed against the 140 kDa holoenzyme *in situ*. Monoclonal antibodies (**B**) bind to the 80 kDa tryptic fragment and the 120 kDa chymotryptic fragment. Monoclonal antibodies (**C**) react with the 35 kDa tryptic fragment and the 120 kDa chymotryptic fragment. The correlation with the known proteolytically cleaved functional parts in the primary structure of the erythrocyte Ca^{2+} pump *in situ* allows to characterize functionally the immunochemical domains. It was suggested that three states **A, B, C** are reflected in different kinetic properties of the erythrocyte Ca^{2+} pump *in situ* (Sarkadi et al. 1988b). Distinction between the individual structural states is a prerequisite for an interpretation of the kinetics measured in the model systems.

Some kinetic aspects

Structural and kinetic data supporting the idea of three states have been reported in studies showing the proteolytically derived conversion of the holoenzyme in situ to 125 kDa, 90 kDa, 81 kDa and 76 kDa phosphorylated fragments (Enyedi et al. 1987; Papp et al. 1989). A calmodulin-like activation, characterized by a high V_{max}, an intermediate Ca^{2+} affinity ($K_{Ca^{2+}}$ approx. 0.4–0.5μmol/l) and a sigmoid calcium activation curve, is produced by trypsinization yielding an 81 kDa phosphorylated fragment. A lipid-like activation, characterized by a high V_{max}, a low calcium affinity ($K_{Ca^{2+}}$ approx. 0.15–0.2μmol/l) and no cooperativity in the calmodulin dependence, is obtained by further trypsinization to yield a 76 kDa phosphorylated fragment. In summary, these results provide evidence that different polypeptide regions of the enzyme are responsible for the regulation (Enyedi et al. 1987). The activation/deactivation cycle of the erythrocyte Ca^{2+} pump *in situ* can be explained in terms of a Ca^{2+}-dependent regulation via calmodulin or calpain, and a Ca^{2+}-independent regulation via acidic lipids and trypsinization (Scheme 1.).

Spatial model

Continued effort is being devoted to analyse the amino acid sequence of (Ca^{2+}+ Mg^{2+})-ATPases. The fragmentation of the purified red cell Ca^{2+} pump with proteolytic enzymes yields small polypeptides; their amino acid sequences are being determined. The ATP-binding site of the fluorescein isothiocyanate-reactive region (Filoteo et al. 1987), a human calmodulin binding domain (James et al. 1988), a human phosphorylation domain (James et al. 1987) as well as the C-terminus of a plasma membrane Ca^{2+}-ATPase from bovine brain (Brandt et al. 1988; Mann et al. 1989) have been sequenced. The cAMP-dependent phosphorylation site



Scheme 1. Different kinetic states of the human red cell membrane Ca^{2+} pump. State **A** is characterized by low V_{max} and high (above 10 μmol/l) $K_{1/2}(Ca^{2+})$; State **B** is characterized by a high V_{max} and intermediate (0.4–0.5 μmol/l) $K_{1/2}(Ca^{2+})$, with a high degree of cooperativity in calcium activation; State **C** represents the fully active enzyme with high V_{max} and low (0.15–0.2μmol/l) $K_{1/2}(Ca^{2+})$. The calcium-dependent and the calcium-independent routes of transitions are indicated. PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PA, phosphatidic acid. (Reproduced from Papp et al. 1989).

located on the C-terminal end of the calmodulin binding domain (Ser 1178) has been confirmed (Neyses et al. 1985; Verma et al. 1988; James et al. 1989b). The amino acid sequences of human erythrocyte (Ca^{2+}+ Mg^{2+})-ATPase isoforms established by protein chemistry and DNA cloning techniques (Verma et al. 1988; Strehler and Carafoli 1988; Strehler et al. 1990) and of rat plasma membrane Ca^{2+} pump isoforms (Shull and Greeb 1988; Greeb and Shull 1989) as well as of

the pig smooth muscle plasma membrane Ca^{2+} pump (De Jaegere et al. 1990) have been reported. Circular dichroism and fluorescence spectroscopy supplied valuable information on the stereochemical situation. The helical content of the pig erythrocyte Ca^{2+}-ATPase decreased by approx. 10% upon transition from the E_1-state to the E_2-state (Krebs et al. 1987). On the basis of the amino acid sequence analysis, cloning techniques and stereochemical information, a spatial model of the erythrocyte (Ca^{2+}+ Mg^{2+})-ATPase has been proposed (Carafoli 1988; Carafoli et al. 1989a,b). This model doubtlessly represents a significant contribution to our understanding of the enzyme structure.

III-C. Conflicting findings concerning the arrangements

III-C.1. The built-in-inhibitor

It is well established that mild proteolysis mimics the effect of calmodulin on the Ca^{2+} pump in red cells (Sarkadi et al. 1980b; Enyedi et al. 1980; Taverna and Hanahan 1980; Niggli et al. 1981a; Stieger and Schatzmann 1981; Adunyah et al. 1982). Calmodulin increases the maximum transport rate (V_{maxB} is approx. 3–5 × V_{maxA}; $V_{\mathrm{maxA}} = 1.2 - 10$ mmol/l cells × $h = 0.3 - 2$ mmol/g membrane protein x h, as measured in ghosts, intact red cells or inside-out membrane vesicles (Romero and Whittam 1971; Schatzmann and Rossi 1971; Schatzmann 1973; Quist and Roufogalis 1975; Sarkadi et al. 1977, 1980a; Macintyre and Green 1978; Szász et al. 1981; Enyedi et al. 1987)). Calmodulin decreases the $K_{Ca^{2+}}$ ($K_{Ca^{2+}A}$ is approx. $3-4\times K_{Ca^{2+}B}$; $K_{Ca^{2+}B}$=10-15μmol/l, incubation media contain unbuffered Ca^{2+} (Sarkadi et al. 1978)). Calmodulin binding to red cell inside-out membrane vesicles treated by trypsin is removed. The idea of a calmodulin-binding domain of the red cell Ca^{2+} pump functioning as a built-in-inhibitor was first suggested by Sarkadi et al. (1980b) and Enyedi et al. (1980). Its action is switched off by calmodulin, mild trypsin treatment and lipid modification. This built-in-inhibitor carries the calmodulin acceptor site, brings about the reduction in affinity of Ca^{2+} sites, e.g. being involved in translocation of Ca^{2+}, and loses its inhibitory effect when calmodulin binds to it. The discrepancies concerning the molecular weight of the calmodulin-binding fragment (Sarkadi et al. 1980b, 1982; Enyedi et al. 1980; Carafoli et al. 1982; Carafoli and Zurini 1982; Wuytack et al. 1982, 1984; McDonald et al. 1982; Benaim et al. 1984; Zurini et al. 1984) could be overcome (Sarkadi et al. 1986a; Zurini et al. 1984):

(i) Electrostatic binding between fragments cleaved off by trypsin exists in SDS-PAGE.

(ii) There is a critical pH value in SDS-PAGE. Associated fragments occur below this value, and they dissociate at higher values.

(iii) Factors such as the type of protein precipitation, composition and pH value of the sample, the amount of the base to neutralize the sample, may influence the association behaviour of digested fragments of (Ca^{2+}+ Mg^{2+})-ATPase during SDS-PAGE. The critical points are pK_a, pH and buffer capacity of buffer substances used for SDS-PAGE. It is up to the individual experimentator to determine which pH and buffer concentrations are suitable to suppress the association of fragments.

Rossi and Schatzmann (1982) showed that in the absence of Ca^{2+} during digestion trypsin has no effect on the calmodulin depleted Ca^{2+}-ATPase activity in red cell inside-out membrane vesicles, whereas in the presence of Ca^{2+} trypsin stimulates this activity. Ca^{2+} was not responsible for the action on trypsin itself. One explanation has been offered that trypsin cleaves off a calmodulin receptor in the absence of Ca^{2+} and in the presence of Ca^{2+} a peptide is removed which contains the calmodulin receptor and an inhibitory sequence. However, Ca^{2+} is not absolutely required for trypsin to be activated and to eliminate the calmodulin stimulation of the erythrocyte Ca^{2+} pump (Sarkadi et al. 1986a).

Experiments with purified erythrocyte Ca^{2+} pump involving limited proteolysis by trypsin favoured a 5 kDa fragment which seems to be fully responsible for calmodulin stimulation, and a 4 kDa fragment containing the calmodulin binding site (Zurini et al. 1984; Benaim et al. 1984, 1986). Experiments with a cleavable radioactive photoaffinity cross-linker (James et al. 1988) and azido-modified calmodulin (Zurini et al. 1984) led to the attractive hypothesis that the purified erythrocyte Ca^{2+} pump carries a second calmodulin binding site with a low affinity. No detailed investigation has as yet been performed to test this assumption.

III-C.2. Oligomerization

Physicochemical evidence for an interaction between the purified erythrocyte, $C_{12}E_8$ soluble, phosphatidylcholine supplemented Ca^{2+}-ATPase molecules has indicated that a transformation from a calmodulin-independent to a calmodulin-dependent form occurs as suggested by kinetic data (Kosk-Kosicka and Bzdega 1988a). Oligomerization replaces the interaction of calmodulin with the Ca^{2+} pump (Kosk-Kosicka and Bzdega 1988b; Kosk-Kosicka et al. 1990). Using radiation inactivation to determine the size of the red cell (Ca^{2+}+ Mg^{2+})-ATPase, the enzyme was found to exist in the dimeric form (Minocherhomjee et al. 1983; Cavieres 1984). A question difficult to answer is whether oligomerization of plasma membrane (Ca^{2+}+ Mg^{2+})-ATPase has any role to play in the transport of Ca^{2+}. In sarcoplasmic reticulum membranes an oligomeric structure of the (Ca^{2+}+ Mg^{2+})-ATPases has often been supposed to be presented (se Michalak 1985; Møller et al. 1988). This idea has been used to explain how the enzyme might function to regulate its full enzymatic activity, which is supported by the fact that approx. 60–70%

of the protein of the sarcoplasmic reticulum membrane is (Ca^{2+}+ Mg^{2+})-ATPase (Martonosi 1969). However, in sarcoplasmic reticulum an oligomeric form of the (Ca^{2+}+ Mg^{2+})-ATPase does not significantly account for changes in enzymatic activity (Le Maire et al. 1976a,b; Jørgensen et al. 1978; Møller et al. 1980).

III-C.3. Lipid requirement

Several experiments have been designed to demonstrate the specificity of the lipid requirement of (Ca^{2+}+ Mg^{2+})-ATPase for its action. As shown in experiments with membrane-bound or purified and reconstituted (Ca^{2+}+ Mg^{2+})-ATPases the same changes in Ca^{2+} affinity and maximal velocity of Ca^{2+} transport as caused by calmodulin and mild proteolytic digestion during the conversion from A to B states are produced by:

- lipid depletion with organic solvents, detergents (Gietzen et al. 1980a,b; Minocherhomjee et al. 1982);
- phospholipase A_2 (Taverna and Hanahan 1980; Al-Jobore and Roufogalis 1981);
- addition of phosphatidylinositol, polyphosphoinositides, phosphatidylserine, lyso-phosphatidylcholine, phosphatidic acid, oleic acid, linoleic acid (Roelofsen and Schatzmann 1977; Ronner et al. 1977; Peterson et al. 1978; Niggli et al. 1979a, 1981a,b; Gietzen et al. 1980a,b; Carafoli et al. 1980a,b, 1982; Al-Jobore and Roufogalis 1981; Niggli and Carafoli 1981; Stieger and Luterbacher 1981; Carafoli and Zurini 1982; Sarkadi et al. 1982; Minocherhomjee et al. 1982; Adunyah et al. 1982). However, phosphatidylcholine, phosphatidylethanolamine or cholesterol did not have corresponding effects, i.e. they had little effect or inhibited the enzyme activity.
- Incubations of red cell inside-out membrane vesicles with phosphatidylinositol-, phosphatidylserine-, phosphatidylcholine-, phosphatidylethanol-amine-, or sphingomyelin-liposomes showed analogous effects as the corresponding lipids without mesophase (Sarkadi et al. 1982).

The experiments have not pointed out to a specific lipid for the (Ca^{2+}+ Mg^{2+})-ATPase to develop its action. However, the molecular basis of information transfer across the protein structure may be influenced by one or more specific signaling lipids. Interesting candidates are the lipids of the phosphatidylinositol cycle (Taverna and Hanahan 1980; Sarkadi et al. 1982; Choquette et al. 1984; Enyedi et al. 1987). It might be that a lipid binding domain of the enzyme confers a binding specificity on the ligand, resulting in an energy minimum immediately after the interaction.

Carafoli and Zurini (1982) proposed that calmodulin, mild proteolysis as well as lipid modification increase the accessibility for the substrates on the active site of the red cell enzyme by conformational transition. It is remarkable that the

experimental findings do not reveal any change in the ATP affinity during the conversion from A to B state and *vice versa*. It may be assumed that only the accessibility to the Ca^{2+}-binding sites is altered. Based on this experiments that lipid modification mimics the action of calmodulin as well as mild the action of proteolysis there may be similarities in the action mechanisms in a general way, e.g. through influencing membrane fluidity. Evidence that the accessibility of active sites changes with membrane fluidity has not been obtained.

III-D. Characteristics of the phosphorylated intermediate formation
III-D.1. Phosphorylation/dephosphorylation

The Ca^{2+} pump can be phosphorylated in the presence of ATP under the influence of Ca^{2+}. Characteristics of the phosphorylated intermediate (EP) formation of the Ca^{2+} pump in red cells are as follows:

- Ca^{2+} is indispensable. The phosphorylation requires micromolar concentrations of Ca^{2+} (Knauf et al. 1972, 1974; Katz and Blostein 1973, 1975; Szász et al. 1978). The concentrations of Ca^{2+} for half-maximal effect are 7 μmol/l in the presence of 125 μmol/l Mg^{2+} (Rega and Garrahan 1975), and 0.2 μmol/l without Mg^{2+} (Szász et al. 1978).
- Mg^{2+} (Katz and Blostein 1975; Rega and Garrahan 1975; Garrahan and Rega 1978; Enyedi et al. 1980; Lichtner and Wolf 1980a,b) and calmodulin (Enyedi et al. 1980; Muallem and Karlish 1980; Rega and Garrahan 1980) accelerate the EP-formation. The halftime for Ca^{2+}-induced EP-formation was 30–40 seconds at 0 °C in a Ca^{2+}-EGTA buffer (Enyedi et al. 1980). Mg^{2+} decreases this value to about 7–10 seconds. Both Mg^{2+} and calmodulin diminish the halftime for Ca^{2+}-induced EP-formation to about 4 seconds.
- The phosphorylated intermediate is acid stable (Rega and Garrahan 1975) and the phosphate bond formed is hydroxalamine sensitive (Katz and Blostein 1975; Rega and Garrahan 1975).

Dephosphorylation occurs predominantly under conditions which cause a rapid decay of the phosphorylated intermediate, i.e. in the presence of high ATP (above 100 μmol/l) together with Mg^{2+}. Luterbacher and Schatzmann (1983) and Schatzmann et al. (1986) have shown that the phosphorylated intermediate decreased slowly when the phosphorylation medium contained only Ca^{2+} and also La^{3+} had been added before dephosphorylation was initiated by 0.5 mmol/l ATP and 1 mmol/l Mg^{2+}. If 1 mmol/l Mg^{2+} was present in the phosphorylation medium, the phosphorylated intermediate rapidly decayed to a very low level. They concluded that La^{3+} interrupts the conformational change of E_1P to E_2P. This conclusion is confirmed by the following experimental fact: In the presence of Ca^{2+} or La^{3+} (E_1-state) the proteolytic digestion of the 80 kDa limit-type fragment *in situ* proceeds rapidly, while EGTA and vanadate (E_2-state) protect the Ca^{2+} pump against proteolytic

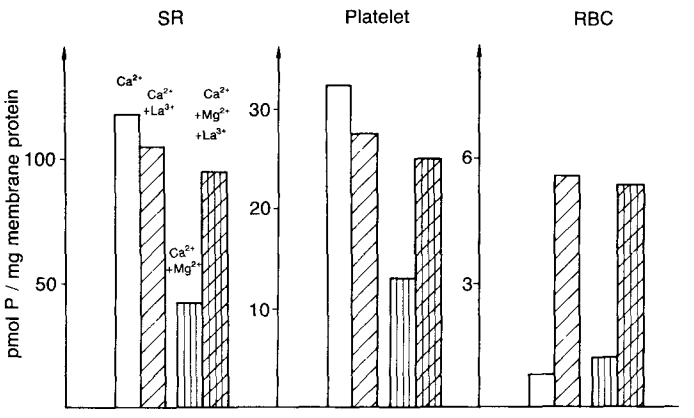


Figure 2. Effects of La^{3+} and Mg^{2+} on the steady state level of the phosphorylated intermediate (EP) in sarcoplasmic reticulum membrane vesicles of rabbit heart muscle (SR), "light" membrane vesicles fraction of human platelets (Platelet) and red cell inside-out membrane vesicles (RBC). Phosphorylation was performed at 4 °C for 30 min in media containing 0.2 μmol/l ATP (including γ^{32}P-ATP), 50 μmol/l $CaCl_2$, or 50 μmol/l $LaCl_3$, or 1 mmol/l $MgCl_2$. Membrane protein concentrations: SR, 0.1 mg/ml; Platelet, 0.4 mg/ml; RBC, 0.8 mg/ml. For explanation see text. (B. Sarkadi, Á. Enyedi personal communication).

degradation (Sarkadi et al. 1986b,c, 1987). In the presence of these latter ligands a retardation of proteolytic destruction is also obtained by using non-specific proteases such as pronase. A similar result was obtained with the purified red cell Ca^{2+} pump (Benaim et al. 1986).

In the isolated human thrombocyte membranes and in the sarcoplasmic reticulum membranes of rabit heart muscle the rate of phosphorylation is substantially higher than the rate of dephosphorylation in the presence of La^{3+} and Mg^{2+} and in the absence of La^{3+} and Mg^{2+} (Fig. 2). In contrast to this, in erythrocyte membranes the rate of phosphorylation is much lower than the rate of dephosphorylation in the absence of La^{3+} and Mg^{2+} but vice versa in the presence of La^{3+} and Mg^{2+} (Fig. 2).

III-D.2. Functions not limited to Ca^{2+} transport

The phosphorylation/dephosphorylation reactions, e.g. the formation of the phosphorylated intermediate, are the means of Ca^{2+} transport function of the enzyme. The pump maintains a transmembrane Ca^{2+} gradient of approx. 10,000 at a permeability of approx. 1 μmol Ca^{2+}/l cells/h in human red cells at 37 °C (Palek et

al. 1976; see Schatzmann 1982). However, the low permeability of the erythrocyte membranes to Ca^{2+} implies that only less than 1% of the ATP production is required for Ca^{2+} transport in intact human red cells (Maretzki et al. 1980). It has been supposed that the human red cell (Ca^{2+}+ Mg^{2+})-ATPase has functions which are not limited to Ca^{2+} transport (Quist and Roufogalis 1975; Sarkadi et al. 1977, 1982; Maretzki et al. 1980, 1981, 1982; Szász et al. 1981; Reimann et al. 1981; Al-Jobore et al. 1981). It is evident from the experiments on human red cells showing:

- a steady state of glycolysis in the presence of membranes only (Maretzki et al. 1977, 1981);
- there are two sites for ATP of the (Ca^{2+}+ Mg^{2+})-ATPase, the one site has a high affinity with a K_m of 2–40μmol/l, and the other site has the low affinity with a K_m of 100-750 μmol/l (Richards et al. 1978; Rapoport et al. 1979; Maretzki et al. 1980, 1981, 1982);
- a bulk of ATP consumption of the membrane in intact cells is due to an ATPase with low affinity for Mg^{2+}-ATP (Maretzki et al. 1980);
- the operation of the red cell Ca^{2+} pump *in vivo* to a small fraction of its capacity. The maximum rate of Ca^{2+} efflux in intact Ca^{2+}-loaded red cells is approx. 85μmol/l cells/min at 37°C, pH 7.4 (Sarkadi et al. 1977);
- phosphatase activity of the Ca^{2+} pump as evident from the *p*-nitrophenyl phosphate cleavage (Pouchan et al. 1969; Garrahan et al. 1970; Rega et al. 1973; see Sarkadi 1980; Caride et al. 1982, 1989; Verma and Penniston 1984; Rossi et al. 1986).

This means that these different activities are not caused by different enzyme entities. However, it was shown that protein kinases and phosphatases operating actively at the inner surface in the presence of Ca^{2+} and Mg^{2+}, are candidates for imitating the Ca^{2+}-ATPase activity (see Szász et al. 1981).

III-D.3. A pattern for the functional classification of Ca^{2+} pumps *in situ*

A synopsis of some selected properties and their relations between these properties of the "erythrocyte type" and the "sarcoplasmic reticulum type" Ca^{2+} pumps *in situ* are shown in Tab. 1. ATPases may be related to the erythrocyte plasma membrane (Ca^{2+}+ Mg^{2+})-ATPase on account of a calmodulin-dependent Ca^{2+}-ATPase/ Ca^{2+} transport and a phosphorylated intermediate or purified enzyme of 140 kDa (Földes-Papp 1985; Minami and Penniston 1987) are those in the plasma membranes of

- intestinal epithelium (De Jonge et al. 1981; Nellans and Popovitch 1981),
- kidney (De Smedt et al. 1981, 1984),
- heart muscle (Caroni and Carafoli 1981),
- brain (Hakim et al. 1982; Papazian et al. 1984),

Table 1. Synopsis of selected properties of the "erythrocyte type" and the "sarcoplasmic reticulum type" (Ca^{2+}+ Mg^{2+})-ATPase *in situ*. ∅: not applicable. (Reproduced from Földes-Papp 1985).

Properties	"Erythrocyte type"	"Sarcoplasmic reticulum type"
Molecular weight (kDa)	140	110
Final phosphorylated, proteolytic products (kDa)	80/76	55,35
Smallest Ca^{2+}-transporting unit (kDa)	80/76	55
Oxalate effect on Ca^{2+}-transporting activity	∅	+(↑)
Calmodulin effect on Ca^{2+}-transporting activity	2–3 times increase	∅
Calmodulin-dependent built-in-inhibitor of Ca^{2+}-transporting activity (kDa)	10	∅
Effect of La^{3+} on steady-state EP-concentration	+(↑)	∅(↓?)
Effect of Mg^{2+} on steady-state EP-concentration	∅(↑)	+(↓)
Ratio of EP-phosphorylation to EP-dephosphorylation rates in the presence of		
Ca^{2+}	< 1	> 1
Ca^{2+} + Mg^{2+}	< 1	< 1
Ca^{2+} + La^{3+}	> 1	> 1

- skeletal muscle (Michalak et al. 1984),
- smooth muscle (De Schutter et al. 1984).

Recently, erythrocyte plasma membrane Ca^{2+} pump epitopes were found in rat kidney distal tubules and human as well as rat placentas (Borke et al. 1989a,b). In rat liver plasma membrane preparations (Lotersztajn et al. 1981; Chan and Junger 1983; Lin and Fain 1984; Bachs et al. 1985) the Ca^{2+}, pump has a molecular weight of approx. 105 kDa and is insensitive to calmodulin activation. It might be a partially proteolysed product, or it may contain an abnormal calmodulin binding domain. The plasma membrane- and the intracellular membrane-associated platelet Ca^{2+}-ATPases have been further characterized by biochemical methods (Enouf et al. 1987, 1988, 1989).

III-E. A problem to Me^{2+}-ATP substrate

Studies with the isolated and purified human erythrocyte Ca^{2+}-ATPase demonstrated that at low ATP (1μmol/l) and Ca^{2+} (0.6μmol/l) concentrations the ATP-

ase activity was inhibited by Mg^{2+} from 5μmol/l up to 1 mmol/l, whereas Mg^{2+} concentrations up to 5μmol/l increased the ATPase activity (Graf and Penniston 1981) The calculated concentrations of Ca^{2+}-ATP strongly decreased or ceased altogether from the point of 5μmol/l In a control experiment at 6 mmol/l ATP and 10μmol/l Ca^{2+} and Mg^{2+} concentrations up to 20 mmol/l did not reveal any inhibitory effect *per se* Under these conditions, Ca^{2+}-ATP was present in saturating concentrations In another experiment, the increase in the ATP hydrolysis was proportional to Ca^{2+}-ATP concentration and it was in inverse relation to free ATP The experimental findings were interpreted as follows Mg^{2+} binds to a special Mg^{2+} site on the enzyme, thereby activating the Ca^{2+}-ATPase, and at low ATP concentrations Mg^{2+} by complexing with ATP removes Ca^{2+}-ATP, which is the substrate for the Ca^{2+}-ATPase

In contrast, revaluation of the energy donor specificity of the Ca^{2+} pump with respect to the *in situ* transport enzyme in red cell inside-out membrane vesicles led to the opposite conclusion (Sarkadi et al 1981) By repeating the experiments of Penniston and co-workers (Penniston et al 1980, Graf and Penniston 1981) with the red cell Ca^{2+} pump *in situ* instead of the isolated and purified human erythrocyte Ca^{2+} pump, the Ca^{2+} transport rate increased at higher Mg^{2+} concentrations (Sarkadi et al 1981) The maximum Ca^{2+} transport rate was observed with total Mg^{2+} concentrations between 200μmol/l and 500μmol/l The suggestion was that Ca^{2+}-ATP is not the energy-donor substrate of the red cell Ca^{2+} pump *in situ* However, the experiments were done in the presence of EGTA It is well established that EGTA shifts the Ca^{2+} affinity of the Ca^{2+} pump to a high value ($K_{Ca^{2+}}$=0 5–1 0μmol/l, Sarkadi et al 1979) The implications of this effect are not yet known It was assumed that the Ca^{2+}-EGTA complex is recognized by the enzyme at a site which is different from the Ca^{2+} site for Ca^{2+} alone Acting on the Ca^{2+}-EGTA binding site and on the Ca^{2+} site for Ca^{2+} alone are necessary for the Ca^{2+} transport (Sarkadi et al 1979) Using EGTA free buffers it was demonstrated that the ATP-dependent Ca^{2+} uptake in red cell inside-out membrane vesicles is stimulated by increasing Mg^{2+}-ATP concentration up to 1 mmol/l (total Mg^{2+}) (Enyedi et al 1982a,b) The Mg^{2+}-ATP dependence in red cell inside-out membrane vesicles was accompanied by a strong decrease of Ca^{2+}-ATP concentration From these findings it was concluded that the true, physiological substrate of the red cell Ca^{2+} pump is Mg^{2+}-ATP

Mg^{2+}-ATP, the energy-donor substrate as suggested by Wolf and co-workers (Wolf 1972, Wolf et al 1977), become controversial The experiments of Schatzmann (1977) were in favour of free ATP Nevertheless, the Mg^{2+}-ATP dependence of the Ca^{2+} pump *in situ* provided evidence for the predominant role of Mg^{2+}-ATP instead of free ATP or Ca^{2+}-ATP It is convenient to regard the red cell Ca^{2+} pump as an enzyme having a relative specificity for Me^{2+}-ATP Furthermore, the purified erythrocyte (Ca^{2+}+ Mg^{2+})-ATPase shows drastic conformational changes

compared with the enzyme *in situ*, which are also indicated by Me^{2+}-ATP substrate specificity. GTP, ITP, CTP and UTP could be substituted for ATP (Lee and Shin 1969; Olson and Cazort 1969). However, in red cell inside-out membrane vesicles the Ca^{2+} pump has a stronger specificity for ATP (Sarkadi et al. 1980a). The experiments on membrane fragments also confirmed these observations (Cha et al. 1971).

Experiments were designed to characterize the metal dependence of the Ca^{2+} pump, with emphasis on the understanding of the mechanisms involved. In red cell inside-out membrane vesicles Mn^{2+}, Co^{2+}, Ni^{2+}, Fe^{2+} activated the ATP-dependent Ca^{2+} transport as a result of forming Me^{2+}-ATP-complexes (Sarkadi et al. 1981; Enyedi et al. 1982b). Concentrations of these divalent metal ions higher than approx. 0.5 mmol/l strongly inhibited Ca^{2+} transport (Enyedi et al. 1982b). These findings were supported by experiments with the phosphorylation as well as the dephosphorylation of the Ca^{2+} pump. At concentrations of approx. 0.5 mmol/l, Mn^{2+} and Ni^{2+} decreased the EP-formation, whereas Co^{2+} and Fe^{2+} inhibited the dephosphorylation (Enyedi et al. 1982b). In the presence of 0.2 mmol/l Mn^{2+}, the $K_{Ca^{2+}}$ increased from about 30 μmol/l to about 60 μmol/l. Co^{2+}, Ni^{2+}, Fe^{2+}, Mg^{2+} did not reduce the Ca^{2+} affinity of the Ca^{2+} pump *in situ* (Enyedi et al. 1982b).

IV. Oxidative Changes

It appears to be a general property of (Ca^{2+}+ Mg^{2+})-ATPases that an imbalance between reactive oxygen species can be assumed to explain a broad range of phenomena relating to these enzymes.

Reversible and irreversible components of inhibition

Thiol- and lipid-dependent mechanisms have been referred to as components which cause inhibition of the erythrocyte Ca^{2+}-ATPase by activated oxygen species (Hebbel et al. 1986; Mankad et al. 1986; Moore et al. 1986). The general finding that Ca^{2+}-ATPases are inhibited by thiol-reactive agents (Richards et al. 1977; Schatzmann and Bürgin 1978; Sarkadi et al. 1980b; Scutari et al. 1980) favours the view concerning the existence of a reversible component relating to the inhibition of the Ca^{2+} pump and reflecting thiol oxidation. Hebbel et al. (1986) observed a disparity among the effects of activated oxygen species on the erythrocyte Ca^{2+}-ATPase. The formation of oxidized glutathione and the oxidation of other thiols by diamide resulted in a reversible decay of the activity of the erythrocyte Ca^{2+}-ATPase. If the decrease of GSH concentration was accompanied by reactions of oxygen radicals, an irreversible component of the inhibition was observed together with a reversible one. The dependences on concentration of oxidants of the reversible and

irreversible components produced on inhibition due to the reaction of oxygen radicals were not measured. Thiol oxidation by PCMB (*p*-chloro-mercuribenzoate) inhibited the Ca^{2+}-ATPase without altering the GSH level. The restoration of Ca^{2+}-ATPase was not GSH-mediated, although GSH was able to break the PCMB metallo-thiol bond. Restoration was obtained by subsequent incubation with DTT (1,4-dithiothreitol). Hebbel et al. (1986) concluded that a portion of the irreversible component relating to the inhibition of the erythrocyte Ca^{2+}-ATPase reflects oxidation of thiols which are inaccessible for reduction by GSH. Indeed, approx. 20% of the erythrocyte membrane sulfhydryl groups are "non-reacting" groups indicated by the lipid soluble reagents diamide, 4,4-dithio-dipyridine and N-ethyl-maleimide and the hydrophilic anionic reagent tetrathionate (Haest et al. 1981). The "non-reacting" sulfhydryl groups are mainly located in the intrinsic proteins (75% in bands 3 and 4.5). As the molecular weight and the solubilization properties of the (Ca^{2+}+ Mg^{2+})-ATPase are similar to those of Band 3 polypeptides (Szász et al. 1978; Niggli et al. 1981b; Graf et al. 1982; Schatzmann 1982), the portion of the irreversible component of inhibition which fails to show any GSH-mediated Ca^{2+}-ATPase recovery could involve "non-reacting" sulfhydryl groups of the (Ca^{2+}+ Mg^{2+})-ATPase located in the hydrophobic core of the red cell membrane. Incubation of normal erythrocytes with 1 mmol/l *t*-butyl hydroperoxide for 30 min at 37°C produced irreversible inhibition of the Ca^{2+}-ATPase (Hebbel et al. 1986). The irreversibility of the inhibition was demonstrated by using 10 mmol/l DTT or 10 mmol/l glucose for subsequent treatment. When erythrocytes were incubated with PCMB prior to exposure to *t*-butyl hydroperoxide and subsequently with DTT or diamide to reverse thiol oxidation, no differences were found in irreversibility of oxidatively inactivated enzyme compared with exposure to *t*-butyl hydroperoxide alone. The conclusion was that lipid peroxidation acts through a non-thiol mechanism. An effect of malonic dialdehyde on the Ca^{2+}-ATPase activity could not be observed (Hebbel et al. 1986; Snider and Moore 1988). However, *t*-butyl hydroperoxide does not only cause lipid peroxidation. Lipid peroxidation and hemoglobin degradation are two extremes of oxidative damage to erythrocytes treated with *t*-butyl hydroperoxide (Trotta et al. 1982). *t*-Butyl hydroperoxide induces oxidation of erythrocyte membrane proteins, produces leaks which are probably due to irreversible protein alteration (Deuticke et al. 1986, 1987a,b), and causes flip sites for the transbilayer reorientation of amphiphatic lipids (Deuticke and Heller 1986; Deuticke et al. 1986). The effects of *t*-butyl hydroperoxide require the formation by hemoglobin of *t*-butoxyl radicals (Trotta et al. 1982) or at least an additional reaction participant such as hemoglobin, hemin, or ferrous chloride with ADP (Leclerc et al. 1988; Moore et al. 1990). The mechanisms of organic hydroperoxide decomposition and the role of solvents in the reactions are not fully understood (Földes-Papp and Maretzki 1982, 1984; Földes-Papp 1983, 1990, Földes-Papp et al. 1981, 1990, 1991; Gerber et al. 1989). There are serious doubts about the identity

of alkoxyl radicals in solution. Alkoxyl radicals have so far escaped direct ESR identification in solution. More recently, studies of the photochemically induced decomposition of *t*-butyl hydroperoxide in DMSO and water have shown that the *t*-butoxyl free radical is involved in the primary process as indicated by direct ESR technique (Földes-Papp 1989, 1990; Földes-Papp et al. 1990, 1991).

Oxidative membrane damage in erythrocytes was shown to proceed after removal of exogenous organic peroxides (Deuticke and Heller 1986; Deuticke et al. 1987a,b). More recently, it was shown that the high-affinity Ca^{2+}-ATPase activity in rat liver plasma membrane is inhibited *in vitro* by 4-hydroxynonenal, a major product of lipid peroxidation, which interacts with sulfhydryl groups (Parola et al. 1990). In view of these observations, oxidative alterations of (Ca^{2+}+ Mg^{2+})-ATPases have received only scant attention. An analysis of regions in the membrane which couple the attack of radicals to structural and functional parts of (Ca^{2+}+ Mg^{2+})-ATPases may clarify some of the physiological processes, whereby toxic chemicals initiate alterations of (Ca^{2+}+ Mg^{2+})-ATPases.

Oxidative and reductive pathways

Experimental findings suggest that the activity of (Ca^{2+}+ Mg^{2+})-ATPases is modulated by alterations in the balance between oxidative and reductive pathways. Moreover, one can assume that this modulation is subject of a complex regulation. A decrease in the calmodulin stimulation of erythrocyte (Ca^{2+}+ Mg^{2+})-ATPase has been observed during red cell aging (Luthra and Kim 1980; Ekholm et al. 1981; Leclerc et al. 1987) as well as in haemolytic anemias such as sickle cell disease (Gopinath and Vincenzi 1979; Dixon and Winslow 1981; Leclerc et al. 1987). The decreased response to calmodulin stimulation in sickle cell disease does not come from the calmodulin activity failure (Dixon and Winslow 1981; Leclerc et al. 1987). Oxidative damage (Hochstein and Jain 1981; Hebbel et al. 1982; Rank et al. 1985; Leclerc et al. 1987) and perhaps selective endogenous proteolysis represent the major factors in the destabilization of the membrane, and are responsible for alterations of the (Ca^{2+}+ Mg^{2+})-ATPase in sickle cell disease. A consequence of cellular changes induced by the sickle anemic conditions may be the increased high-affinity Ca^{2+} binding by the red cell membrane (Litosch and Lee 1980). Differences in functional properties of the erythrocyte membrane induced by heating canine blood to 57 °C (thermal trauma) have been attributed to changes in membrane ATPase activity levels rather than to changes in intraerythrocytic Ca^{2+} levels (Hilton 1986). Studies with glucose-6-phosphate-dehydrogenase-deficient red cells indicated the dependence of the (Ca^{2+}+ Mg^{2+})-ATPase activity on the cell redox status (Shalev et al. 1985). Monitoring of the hypertension disease is possible by estimation of the red cell Ca^{2+}-ATPase activity (Syurin et al. 1990).

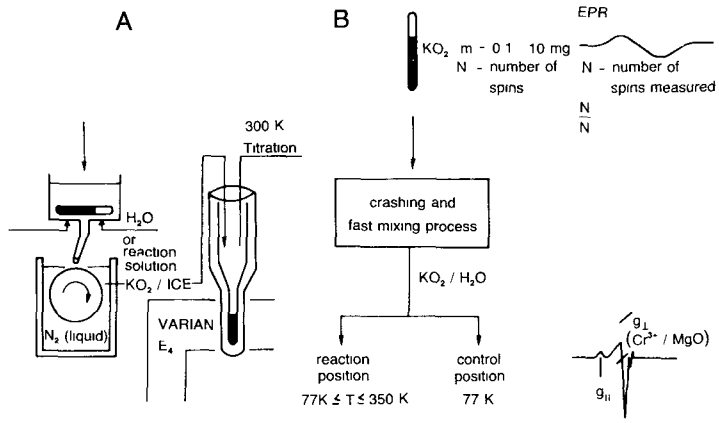


Figure 3. A glass ampulla containing a known amount of KO_2 was introduced into the mixing chamber and crashed. The KO_2 was mixed with water, distributed finely by a nozzle and projected with high velocity on a metallic specially coated roll rotating in liquid nitrogen; The duration from crushing to freezing never exceeded 0.5 seconds. The frozen debris was collected and analysed at 77 K in the ESR spectrometer (model E_4, operating at the X-band; Varian, Palo Alto, CA, USA). By using different amounts of KO_2 (0.1–10 mg) and by varying the distances of the control and reaction position from mixing chamber, a desired starting concentration of O_2^- can be adjusted. OH radicals generated in secondary reactions are amenable by that procedure, too.

Direct methods for investigation of radical reactions

A great deal of methods by which oxygen radicals were determined have been based upon electron transfer and hydrogen abstraction properties of these species. The disadvantages of indirect methods used for free radical detection (Földes-Papp et al. 1989a) are:

- dependence on initiation, competition or inhibition in indicator systems;
- secondary reactions in indicator systems influence the detection of radicals;
- low specificity of indicator systems.

The disadvantages are avoided by direct methods for investigations of radicals. To clear up stabilization phenomena of O_2^- in aqueous media (Földes-Papp 1986), O_2^- was detected by rapid freezing to 77K and ESR. An attempt was made to find out the conditions which considerably contribute to the well defined accurate, reproducible handling of O_2^- and OH in aqueous system. The technique developed is schematically shown in Fig. 3. The features of the technique (Földes-Papp et al. 1988a) are:

- transport of O_2^- in an aqueous medium,

- proportional sharing of the solution into the reaction and control position,
- parallel and synchronous determination of O_2^- by rapid freezing to 77K and ESR.

It is commonly accepted that the O_2^- radical in aqueous media has a life-time in the range of milliseconds. ESR studies in a KO_2/H_2O/iron ion system revealed a stabilization of a part of the initially added O_2^- lasting up to hours at 300K (Földes-Papp et al. 1988a,b,c, 1989a,b; Földes-Papp 1990), to our knowledge, this is the first report of this kind. In these ESR studies, the nature of this effect was investigated. The iron-mediated long-term stabilization of O_2^- in an aqueous system is based on the existence of an oxidic iron hydrate phase with a partially mixed-valence character. It was also shown to be present in aqueous systems with low iron ion content (1 μmol/l). Besides physical adsorption phenomena favoured by the charge of O_2^- a more mechanistic interpretation should also consider electronic interactions (Földes-Papp et al. 1989c). Species like $[Fe^{+++}(H_2O)_xOH_y^-]_z$ O_2^- may exist. On this basis the conclusion is drawn that the reactivity and selectivity of O_2^- is modified by specific interaction (adsorption) and binding interaction (electron exchange) on other chemical structures (Földes-Papp 1990). The differentiation of the reaction behaviour, e.g. the possibility of O_2^- to find new routes of reactions, is expected to increase in biological systems. Therefore, the special biological structure is very important in the modification of reactivity and selectivity of O_2^-. In further studies it must be investigated whether the iron mediated long-term stabilization of O_2^- in aqueous systems has effects on (Ca^{2+}+ Mg^{2+})-ATPases. O_2^- adsorbed on an oxidic iron hydrate phase in aqueous systems might function as a strong oxidant similar to that species which has been suggested to be a complex between oxygen and different valence states of iron in the initiation of lipid peroxidation by Fe^{2+} and hydrogen peroxide (see Minotti and Aust 1987).

V. Conclusions

The three-dimensional structure of animal and human plasma membrane (Ca^{2+}+ Mg^{2+})-ATPases is not yet known. The progress in methodology and new aspects of (Ca^{2+}+ Mg^{2+})-ATPase research will help us to understand that the two-dimensional structure in near future. Nevertheless, biochemical and immunological methods as well as studies using radical reactions have been valuable tools for the elucidation of the molecular structure. As illustrated in the review, this was the way leading to detailed understanding in recent years.

Acknowledgements. The work reported in this article was supported by Professor Dr. George Gárdos, Professor Dr. Balázs Sarkadi of the National Institute of Haematology and Blood Transfusion in Budapest, and Dozent Dr. Reinhard Stösser of the Department of Chemistry at the Humboldt University in Berlin. I thank Professor Dr. Hartmut

Seliger, Dr. José Flávio Ramalho-Ortigão, Dr. Gabriele Gröger, and Mrs. Ingelore Spitzberg of the Section of Polymers at the University in Ulm for helpful comments. I am indebted to Dr. Kailash Chand Gupta—on leave from Nucleic Acids Reserch Laboratory, CSIR Centre for Biochemicals, Delhi University Campus, Delhi—for critically reading the manuscript.

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Final version accepted August 1, 1991