Chaotropic Agents and Increased Matrix Volume Enhance Binding of Mitochondrial Cyclophilin to the Inner Mitochondrial Membrane and Sensitize the Mitochondrial Permeability Transition to [Ca^{2+}] †

Cathal P. Connern and Andrew P. Halestrap*

Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

Received October 23, 1995; Revised Manuscript Received March 15, 1996⊗

ABSTRACT: Binding of mitochondrial cyclophilin (CyP) to the inner mitochondrial membrane is induced by treatment of mitochondria with thiol reagents or oxidative stress and correlates with a sensitization to [Ca^{2+}] of the cyclosporin A-sensitive mitochondrial permeability transition pore (MTP) [Connern, C. P., & Halestrap, A. P. (1994) *Biochem. J. 303*, 321−324]. Here we show that detection of the bound CyP by Western blotting is greatly enhanced by fixing the CyP to the blotting membrane with glutaraldehyde. CyP binding was only observed when mitochondria were incubated and then frozen in KSCN medium before preparation of the membrane fraction, but not when KCl medium was used. However, incubation of mitochondria (energized or deenergized) in KCl medium followed by KSCN addition immediately prior to freezing did allow CyP binding to be detected. The action of KSCN could be mimicked by guanidinium chloride, implying that the chaotropic action of these agents stabilized the bound complex. The sensitivity to [Ca^{2+}] of the MTP in deenergized mitochondria was greatly enhanced in KSCN medium as compared to KCl medium. Binding of CyP to the mitochondrial membrane was increased by treatment with *tert*-butylhydroperoxide, phenylarsine oxide, and diamide and by hypoosmotic KCl medium. These conditions all increased the sensitivity of the MTP to [Ca^{2+}]. Conditions known to increase the mitochondrial NADH/NAD^+ ratio decreased CyP binding. In contrast, the effects of mitochondrial membrane potential, matrix pH, and adenine nucleotide translocase conformation on the sensitivity of the MTP to [Ca^{2+}] were not associated with a change in CyP binding. Our data imply that there may be two independent mechanisms of altering the Ca^{2+} sensitivity of the MTP, one brought about by CyP binding which is stabilized by chaotropic agents and another involving additional regulatory sites on the pore complex.

The mitochondrial matrix contains about 50 pmol/mg of mitochondrial protein of a distinct 18.5-kDa isoform of cyclophilin (CyP).[1] Like other cyclophilins, this acts as a peptidyl−prolyl *cis*−*trans* isomerase (PPIase) that is inhibited by the immunosuppressant drug cyclosporin A (CsA) with a K_i of about 5 nM (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991; Connern & Halestrap, 1992). N-Terminal sequencing of the purified CyP has provided strong evidence that it is identical to the human CyP3 gene product and undergoes translocation into the mitochondrial matrix before removal of its presequence (Bergsma *et al*., 1991; Connern & Halestrap, 1992). It is now more usually called CyP-D. The immunosuppressive action of CsA is mediated by a complex between cytosolic CyP (CyP-A) and CsA that inhibits calcineurin, a Ca-sensitive protein phosphatase. This, in turn, leads to a block of the Ca-dependent signal transduction pathway of T-lymphocyte activation (Schreiber & Crabtree, 1992; Fruman *et al*., 1994; Galat & Metcalfe, 1995). However, the natural function of the cyclophilins is still uncertain, although their PPIase activity and their presence in subcellular organelles suggests that the folding of proteins, both newly synthesized and following translocation into a subcellular compartment such as the endoplasmic reticulum (ER) and mitochondria, may be an important role (Schmid, 1993; Fruman *et al*., 1994; Galat & Metcalfe, 1995). Indeed, CsA has been shown to slow the synthesis of secreted proteins and cause their accumulation in the ER (Steinmann *et al*., 1991; Lodish & Kong, 1991; Colley *et al*., 1991). It also prevents proper cell surface expression of oligomeric ligand-gated ion channels (Helekar, 1994) and delays protein refolding within mitochondria of *Neurospora crassa* (Rassow *et al*., 1995).

When mitochondria are exposed to supraphysiological concentrations of Ca^{2+}, they become nonselectively permeable to small molecules (<1500 Da). This permeability transition is greatly sensitized to [Ca^{2+}] by oxidative stress, adenine nucleotide depletion, elevated phosphate concentrations, low membrane potential, and agents that stabilize the C conformation of the adenine nucleotide translocase (ANT). In contrast, protection is afforded by low pH, high membrane

† This work was supported by the Medical Research Council.

* Address correspondence to this author.

⊗ Abstract published in *Advance ACS Abstracts,* June 1, 1996.

[1] Abbreviations: AMPA, α-amino-3-hydroxy-5-methylisoxazolepropionic acid; ANT, adenine nucleotide translocase; BSA, bovine serum albumin; CyP, cyclophilin; CsA, cyclosporin A; ECL, enhanced chemiluminescence; EGTA, ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ER, endoplasmic reticulum; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FKBP, FK506 binding protein; Mops, 3-morpholinopropanesulfonic acid; MTP, mitochondrial permeability transition pore; NAD^+ and NADH, nicotinamide adenine dinucleotide, oxidized and reduced forms; NTA, nitrilotriacetic acid; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol); PheArs, phenylarsine oxide; PMSF, phenylmethanesulfonyl fluoride; PPIase, peptidyl−prolyl *cis*−*trans* isomerase; SMP, submitochondrial particles; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBH, *tert*-butylhydroperoxide; TGF, tumor growth factor; Tris, tris(hydroxymethyl)aminomethane.

S0006-2960(95)02517-7 CCC: $12.00

potential, and agents that stabilize the M conformation of the ANT (Crompton *et al*., 1987; Gunter & Pfeiffer, 1990; Bernardi *et al*., 1994; Halestrap, 1994). In addition, pore opening can be prevented by submicromolar concentrations of CsA and its analogues (Crompton *et al*., 1988; Broekmeier *et al*., 1989; Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991). This effect appears to be mediated through the matrix cyclophilin since the affinity of this protein for a number of CsA analogues correlates with their ability to inhibit pore opening (Griffiths & Halestrap, 1991; Nicolli *et al*., 1996). These observations, and studies by other (Bernardi *et al*., 1994; Petronilli *et al*., 1994b), have led to the suggestion that an interaction of CyP with an integral membrane protein leads to a change in its conformation which, when triggered by Ca^{2+}, induces pore opening. In view of the influence of the ANT conformation on this process, we suggested that the integral membrane protein might be the ANT (Halestrap & Davidson, 1990; Griffiths & Halestrap, 1991). More recently we have shown that oxidative stress and thiol reagents that enhance pore opening recruit CyP to the inner mitochondrial membrane, which is consistent with this model (Connern & Halestrap, 1994).

In this paper we show that the chaotropic agents thiocyanate and guanidinium or an increase in the mitochondrial matrix volume enhance the binding of CyP to the inner mitochondrial membrane. These effects are associated with an increased sensitivity of the pore to $[Ca^{2+}]$ and provide additional evidence that binding of CyP to a target protein is important in pore formation. It is suggested that the CyP−membrane protein complex may be stabilized by chaotropes, as has been described for some receptor−agonist complexes (Maksay & Ticku, 1984; Honoré & Nielsen, 1985; Honoré & Drejer, 1988; Hall *et al*., 1992, 1993).

EXPERIMENTAL PROCEDURES

Materials

Rat liver mitochondria were prepared as described by Halestrap and Davidson (1990) and either used within 6 h (fresh mitochondria) or stored on ice overnight in isolation buffer at a concentration of 50−80 mg of protein/mL (aged mitochondria). We have demonstrated previously that fresh and aged mitochondria behave similarly with respect to CyP binding and the Ca^{2+} sensitivity of pore opening (Connern & Halestrap, 1994). For some experiments, mitochondria were depleted of their endogenous adenine nucleotides by incubation for 15 min at 25 °C in sucrose isolation buffer supplemented with 2 mM PP_i as described previously (Griffiths & Halestrap, 1995). N-Terminal anti-peptide antibodies to the mature mitochondrial cyclophilin were raised and purified as described elsewhere (Connern & Halestrap, 1994). KSCN was obtained from BDH/Merck Ltd. (Lutterworth, Leicestershire, U.K.) and guanidinium chloride from Sigma Chemical Co. (Poole, Dorset, U.K.). The sources of all other chemicals and biochemicals were as previously described (Halestrap & Davidson, 1990; Halestrap, 1991; Connern & Halestrap, 1994).

Methods

Measurements of Pore Opening: (A) Mitochondrial Swelling. Mitochondria were usually incubated at 25 °C and 0.5 mg of protein/mL in 3.5 mL of buffer (pH 7.2) containing 20 mM Mops, 10 mM Tris, 2 mM nitrilotriacetic acid (NTA), 0.5 μM rotenone, 0.5 μM antimycin A, 2 μM A23187, and either 150 mM KSCN or 150 mM KCl. The calcium ionophore A23187 was added to ensure complete equilibration of Ca^{2+} across the mitochondrial inner membrane under deenergized conditions (Halestrap, 1991). A_{520} was monitored continuously in a spectrophotometer with computerized data acquisition and averaging as described previously (Halestrap & Davidson, 1990). Mitochondrial pore opening is associated with swelling that is accompanied by a decrease in light scattering detected as a decrease in A_{520}. Maximal rates of swelling were determined by differentiation of the traces.

(B) Shrinkage of Preswollen Mitochondria. Mitochondria were preswollen as described previously (Connern & Halestrap, 1994) by incubation at 3 mg of protein/mL for 20 min at 30 °C in the KSCN buffer described above, but without added A23187 or NTA and with the addition of 1 mM $CaCl_2$. Additions of *tert*-butylhydroperoxide (TBH), diamide, or phenylarsine oxide (PheArs) to the buffer were made as required. Swelling was terminated by addition of 1.2 mM EGTA and centrifugation at 12000*g* for 10 min to sediment the swollen mitochondria, which were then resuspended at 3 mg of protein/mL in either KSCN or KCl buffer (again without NTA or A23187). In order to ensure equilibration of the matrix with the KCl medium, the swollen mitochondria were incubated again at 30 °C in the new medium supplemented with 1 mM $CaCl_2$. After 2 min, 1.2 mM EGTA was added and the mitochondria were sedimented by centrifugation before resuspending at 30 mg protein/mL in either KSCN or KCl buffer (now with added NTA and A23187). The extent of pore opening at different $[Ca^{2+}]$ was determined by the shrinkage of mitochondria upon addition of 7% (w/v) poly(ethylene glycol) (PEG) as described previously (Connern & Halestrap, 1994). Briefly, preswollen mitochondria (approximately 1 mg of protein) were added to a cuvette containing 3 mL of KSCN or KCl buffer at 25 °C supplemented with $CaCl_2$ to give the desired free $[Ca^{2+}]$. A_{520} was continuously monitored (10 data points/s), and after 1 min, 0.5 mL of 50% (w/v) PEG 2000 was added through a light-sealed injection port and rapidly mixed (<1 s). Shrinkage induced by the colloidal osmotic pressure of the PEG was detected as a rapid increase in A_{520}, whose rate was determined by differentiation.

Cyclophilin Binding. Routinely, aged mitochondria were incubated at 25 °C and 0.5 mg/mL mitochondrial protein in 10 mL of complete KSCN or KCl buffer with the addition of protease inhibitors antipain, pepstatin, and leupeptin at 1 μg/mL and PMSF at 100 μM. Cyclophilin binding was initiated by the addition of 1 mM TBH, 0.1 mM diamide, or 0.1 mM PheArs. After 30 s of incubation, the samples were frozen in liquid nitrogen and membranes prepared by freeze/thawing as described previously (Connern & Halestrap, 1994). For determination of CyP binding under energized conditions, the protocol was similar, but mitochondria were incubated in 125 mM KCl, 20 mM Mops, and 10 mM Tris containing 0.2 μM rotenone, 2.5 mM potassium phosphate, 5 mM Tris succinate, and 0.5 mM EGTA, pH 7.2, with protease inhibitors added as above. Any variations to this protocol are described in the appropriate figure legends. For experiments performed in KCl media, the effects of chaotropic agents (150 mM KSCN or guanidinium chloride) were studied by adding them to the incubation immediately before

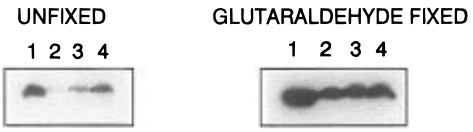

FIGURE 1: Effects of glutaraldehyde fixation on the detection of cyclophilin by Western blotting. Rat liver mitochondria (aged) were incubated at 25 °C and 3 mg of protein/mL of KSCN medium (see Methods) containing protease inhibitors and, where appropriate, 1 mM TBH or 0.1 mM diamide. After 1 min, membranes were prepared by freezing and thawing and proteins separated by SDS−PAGE (20 μg/lane) and transferred to Immobilon-P membranes using a semidry blotter. Samples were blocked with 10% milk powder either immediately (unfixed) or after the fixation of samples onto the membrane with glutaraldehyde (see Methods). Bound cyclophilin was detected using an anti-peptide antibody to mitochondrial cyclophilin and ECL detection. Lanes 2−4 represent membranes from control, 1 mM TBH-treated, and 0.1 mM diamide-treated mitochondria, respectively, while lane 1 contained 12 μg of partially purified matrix CyP.

freezing in liquid nitrogen. Detection of CyP binding to the membranes was performed using SDS−PAGE and Western blotting with N-terminal anti-peptide antibodies to mitochondrial CyP as described previously (Connern & Halestrap, 1994) but with one critical modification. After blotting onto Immobilon-P membranes, the samples were fixed onto the membrane by incubation for 10 min in 0.1% Tween−PBS buffer containing 0.05% glutaraldehyde. (Diggle *et al*., 1995). The membranes were then washed with PBS−Tween buffer and blocked with 10% milk powder before detection of bound cyclophilin with the CyP antibodies using an enhanced chemiluminescence (ECL) detection kit (Amersham, Buckinghamshire, U.K.). For SDS−PAGE, 10−20 μg of protein (determined by Bradford assay using BSA as standard) was routinely loaded per lane. Western blotting with antibody to the mitochondrial NAD^+-dependent isocitrate dehydrogenase (raised in chicken and generously provided by Professor R. M. Denton of this department) was also performed in parallel to correct for changes in nonspecific CyP binding. Detection was by enhanced chemiluminescence (ECL).

RESULTS

Glutaraldehyde Fixation Prevents Loss of CyP from Immobilon Membranes. Cyclophilin binding to mitochondrial membranes was shown previously to be dependent upon oxidative stress and thiol modification (Connern & Halestrap, 1994). However, the detection of CyP by Western blotting appeared to vary considerably from one experiment to the next. We have now demonstrated that this is due to variable loss of CyP from the blotting membrane during blocking with milk powder and subsequent washing of the membrane. A similar problem was noted by Diggle *et al*. (1995), who found that treatment of the blotting membrane with 0.05% glutaraldehyde immediately after protein transfer could be used to retain small proteins on Immobilon during blotting. The data of Figure 1 show that this procedure also works for CyP. In the unfixed samples little CyP was detected in the controls, while TBH and diamide gave appreciable binding as reported previously (Connern & Halestrap, 1994). In contrast, in the fixed samples at the same protein labeling and film exposure, considerable CyP binding was detected even in the controls. Indeed, the effect of the TBH and diamide appeared to be lost, but this was a result of overexposure of the film. In subsequent experiments glut-

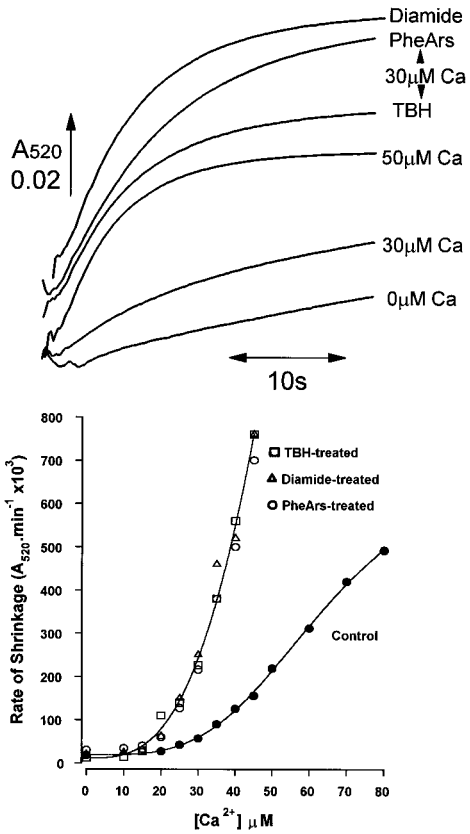

FIGURE 2: Effects of thiol reagents and oxidative stress on the Ca^{2+} sensitivity of the mitochondrial permeability transition pore. Aged mitochondria were preswollen in KSCN buffer containing, where appropriate, 1 mM TBH, 0.1 mM diamide, or 20 μM phenylarsine oxide (PheArs) and reisolated as described under Methods. The extent of pore opening at varying $[Ca^{2+}]$ was investigated using the rate of shrinkage induced by addition of 7% (w/v) PEG. In the top panel, typical traces are shown, while in the bottom panel, initial rates of shrinkage at a range of $[Ca^{2+}]$ are plotted for each treatment. Data are shown of one experiment typical of at least three.

araldehyde fixation was used routinely, less protein was loaded per lane for SDS−PAGE, and a greater antibody dilution was used in Western blotting. Under these conditions, a substantial increase in binding of CyP in response to treatment of mitochondria with 1 mM *tert*-butylhydroperoxide (TBH), 0.1 mM diamide, or 0.1 mM phenylarsine oxide (PheArs) could readily be observed (e.g., Figures 3−5). In five separate experiments, scanning of the blots demonstrated that the binding of CyP in the presence of the three agents, expressed as a percentage of control (± S.E.M.), were 286% ± 36%, 573% ± 118%, and 288% ± 34%, respectively. Diamide consistently gave the greatest binding and from parallel blots of a mitochondrial matrix fraction we estimated that about 50% of the total matrix CyP (50 pmol/mg of mitochondrial protein; Connern & Halestrap, 1992) was bound under such conditions. No consistent changes in the binding of NAD^+-dependent isocitrate dehydrogenase were detected following any of the treatments (see Figure 3).

Comparison of the CyP Binding and Pore Opening Induced by Oxidative Stress and Thiol Reagents in KSCN and KCl Media. In parallel with the determination of CyP binding in KSCN medium described above, measurements of the sensitivity of pore opening to $[Ca^{2+}]$ were also made, and results of a typical experiment are shown in Figure 2. For this purpose mitochondria were treated with 1 mM TBH,

0.1 mM diamide, or 20 μM PheArs before swelling with 1 mM $CaCl_2$ to produce preswollen mitochondria for use in the PEG-induced shrinkage assay. This allowed determination of the extent of pore opening at increasing [Ca^{2+}]. Typical traces are shown in the top panel, while rates of shrinking at increasing [Ca^{2+}] are shown in the bottom panel. The effect of all the treatments consistently produced a similar sensitization of the pore opening to [Ca^{2+}] despite their different effects on CyP binding reported above. These results suggest that if CyP binding to a membrane protein is responsible for the increased sensitivity to [Ca^{2+}], this target protein may be present in excess of other proteins participating in pore formation. Our previous suggestion (Halestrap & Davidson, 1990) of the involvement of the adenine nucleotide translocase (ANT) in pore formation would be compatible with this model since liver mitochondria contain some 400 pmol of ANT/mg of protein (Klingenberg, 1976), well in excess of CyP.

We have routinely studied pore opening in KSCN buffer since it was shown to give the most reproducible results (Halestrap & Davidson, 1990). However, we were interested in determining the effect of mitochondrial membrane potential on CyP binding, since Bernardi and colleagues have provided strong evidence that reagents that sensitize the pore to [Ca^{2+}] do so by antagonizing the inhibitory effect of the membrane potential (Bernardi, 1992; Petronilli *et al*., 1993a,b, 1994a). It is not possible to energize mitochondria in 150 mM KSCN since this medium inhibits the respiratory chain (A. P. Halestrap, unpublished data). Thus we changed to a KCl medium which enabled mitochondria to be incubated under deenergized (rotenone + antimycin) or energized (rotenone + succinate) conditions. When mitochondrial membranes were prepared following such incubations, no CyP binding was detected even after diamide treatment (see Figure 3, lanes 7−12). This was the case whether mitochondria were deenergized (Figure 3) or energized (data not shown). However, if 150 mM KSCN was added immediately prior to freezing the mitochondria in liquid nitrogen, CyP could be detected in the membrane fraction of both energized (Figure 4) and deenergized (Figure 3) mitochondria. Binding was increased by treatment with TBH, diamide, or PheArs in a similar fashion to that observed when the treatments were performed in KSCN medium. No consistent difference in the levels of NAD^+-dependent isocitrate dehydrogenase detectable in the membrane fraction was shown either by addition of the KSCN or by any of the treatments (Figure 3). Energization itself also appeared to exert no effect on CyP binding since the addition of uncoupler to energized mitochondria did not cause a significant change in the amount of bound CyP (Figure 4, lane 5).

In order to confirm that there was no effect of energization of CyP binding, we also compared CyP binding in mitochondria incubated in standard KCl medium containing rotenone and antimycin (deenergized) with those in the same medium in which antimycin was replaced by 5 mM succinate (energized). In six separate experiments, CyP binding in the presence of succinate was 39.7% ± 6.7% that in the presence of antimycin. These data might be taken to contradict the data with FCCP. However, in the presence of rotenone, succinate increases the $NADH/NAD^+$ ratio in the matrix in addition to energizing the mitochondria (Rigobello *et al*., 1995). There is strong evidence that

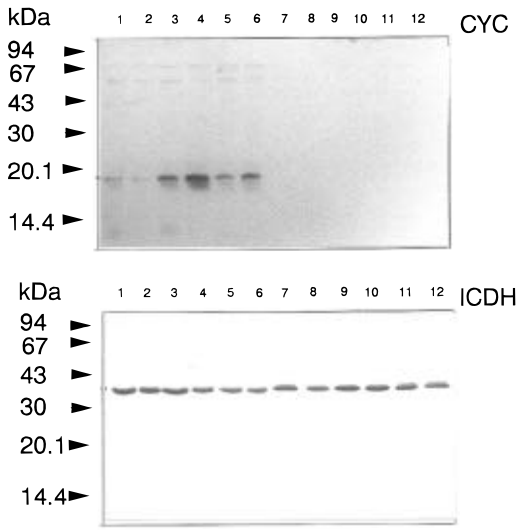

FIGURE 3: Effects of thiol reagents and oxidative stress on CyP binding to mitochondrial membranes of deenergized mitochondria incubated in KCl medium. Mitochondria were incubated at 25 °C and 0.5 mg of protein/mL in 9 mL of KCl medium (see Methods) containing both rotenone and antimycin at 0.5 μg/mL and further additions as follows: none (lanes 1 and 7), 0.2 mM $CaCl_2$ (lanes 2 and 8), 1 mM TBH (lanes 3 and 9), 0.1 mM diamide (lanes 4 and 10), 0.02 mM phenylarsine oxide (PheArs; lanes 5 and 11), or 0.1 mM PheArs (lanes 6 and 12). After 30 s the mitochondrial mixture was either immediately frozen in liquid N_2 (lanes 7−12) or frozen after addition of 1 mL of 1.5 M KSCN, 200 mM Mops, and 100 mM Tris, pH 7.2 (lanes 1−6). After 5 cycles of freeze/thawing, membranes were prepared and subjected to SDS−PAGE (12 μg of protein/lane) and Western blotting as described under Methods. In the top panel blotting was with anti-CyP antibody, while in the bottom panel anti-ICDH antibody was used.

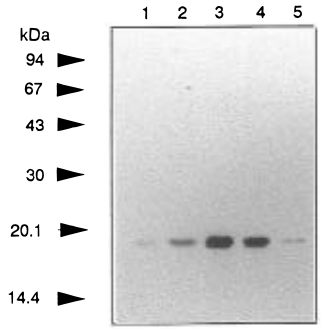

FIGURE 4: Effects of thiol reagents and oxidative stress on CyP binding to mitochondrial membranes of energized mitochondria incubated in KCl medium. Incubations were performed as described in the legend to Figure 3 except that the KCl medium contained 2 mM succinate in place of antimycin. Treatment of mitochondria with KSCN before preparation of membranes by freeze/thawing and analysis by SDS-PAGE and Western blotting were performed as described in Figure 3. Additions to the KCl incubation medium were none (lane 1), 1 mM TBH (lane 2), 0.1 mM diamide (lane 3), 0.1 mM PheArs (lane 4), or 1 μM FCCP (lane 5).

oxidation of matrix $NADH/NAD^+$ can sensitize pore opening to [Ca^{2+}], probably by affecting protein thiol status (Hunter & Haworth, 1979; Rigobello *et al*., 1995; Chernyak *et al*., 1995). To establish whether the $NADH/NAD^+$ ratio might affect CyP binding we used conditions similar to those employed by Rigobello *et al*. (1995) to correlate protein thiol oxidation with mitochondrial pore opening. In the presence of rotenone with deenergized mitochondria, addition of 1 mM pyruvate decreased CyP binding to 23.9% ± 4.7% (n = 3) of the control value, while in a single experiment

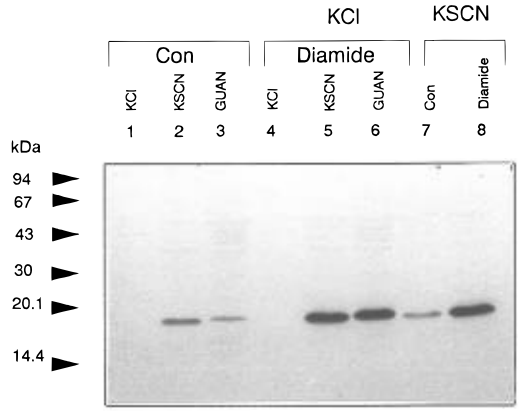

FIGURE 5: Effect of guanidinium chloride on CyP binding to mitochondrial membranes. For lanes 1−6, aged mitochondria were incubated at 8 mg of protein/mL under deenergized conditions in a KCl medium in the presence (lanes 4−6) or absence (lanes 1−3) of 0.1 mM diamide as indicated. After 30 s of incubation at 25 °C, 1 mL of the incubation medium was diluted into 9 mL of medium containing 20 mM Mops, 10 mM Tris, 0.5 μM antimycin, 0.5 μM rotenone, protease inhibitors, and either 150 mM KCl (lanes 1 and 4), 150 mM KSCN (lanes 2 and 5), or 150 mM guanidinium chloride (lanes 3 and 6) and immediately frozen in liquid N_2. For lanes 7 and 8 incubations were carried out at 0.8 mg of protein/mL in KSCN medium in the presence (lane 8) and absence (lane 7) of 0.1 mM diamide before freezing. Membranes proteins were separated by SDS−PAGE (12 μg of protein/lane), and CyP was detected by Western blotting.

addition of 5 mM L-glutamate + 2 mM L-malate caused a decrease to 47% of the control value. Thus intramitochondrial NADH may indeed exert its inhibitory effect on pore opening by decreasing CyP binding.

Effect of Chaotropic Agents on CyP Binding and Sensitivity of Pore Opening to [Ca^{2+}]. Our data indicate that KSCN either enhances or stabilizes binding of CyP to the mitochondrial membrane. Since KSCN is a powerful chaotropic agent (Hatefi & Hanstein, 1973), it was of interest to see whether another chaotropic agent, guanidinium chloride, exerted a similar effect. The data of Figure 5 show that this is the case. In these experiments deenergized mitochondria were preincubated in KCl medium at 8 mg of protein/mL in the presence and absence of diamide and then diluted 10-fold into isoosmotic KSCN or guanidinium chloride medium before freezing and preparation of membranes in the usual manner. Since the thiocyanate and guanidinium are of opposite charges, it is most likely that it is their chaotropic properties rather than an alteration of the membrane surface charge that is responsible for their effect (McLaughlin, 1975).

If CyP binding is important in regulating the Ca^{2+} sensitivity of the pore, then the enhanced CyP binding that occurs in KSCN medium should be accompanied by enhanced sensitivity of pore opening to [Ca^{2+}]. This was investigated using both swelling and shrinking assays, and the data are reported in Figures 6−8. When the swelling assay was carried out with control mitochondria in KCl medium, no pore opening was observed until >200 μM Ca^{2+} was added (Figure 6), whereas in KSCN medium significant pore opening was observed at 50 μM Ca^{2+}. In both media the sensitivity to [Ca^{2+}] was increased by either diamide or TBH treatment. In order to increase the sensitivity of pore opening to [Ca^{2+}] and to avoid variations in the response caused by variable concentrations and phosphorylation states of intramitochondrial adenine nucleotides, some mitochondria were pretreated with 2 mM PP_i, which removes >90% of their adenine nucleotides (Novgorodov *et al*., 1992; Griffiths & Halestrap, 1995). These mitochondria were more sensitive to [Ca^{2+}] as expected (dashed versus solid lines in Figure 6) and the sensitivity remained greater in KSCN medium than KCl medium. Maximal rates of swelling at increasing [Ca^{2+}] in both media are summarized in Figure 7. In both cases, diamide and TBH treatment of PP_i-treated mitochondria enhanced the sensitivity of pore opening to [Ca^{2+}], although the effect was less pronounced than with control mitochondria (Figure 6). The effect of KSCN to enhance the sensitivity of pore opening to [Ca^{2+}] was confirmed in the PEG-induced shrinkage assay as illustrated in Figure 8. In these experiments mitochondria were preswollen in KSCN medium before equilibration in either KSCN or KCl medium for measurement of the rate of shrinkage upon addition of 7% PEG at increasing [Ca^{2+}]. Once again, the mitochondria in KCl medium were significantly less sensitive to Ca^{2+} than those in KSCN medium.

Effects of Mitochondrial Matrix Volume on CyP Binding and Ca^{2+} Sensitivity of Pore Opening. We have noticed previously that when deenergized mitochondria are incubated in sucrose medium, pore opening is sensitized to [Ca^{2+}] if the osmolarity of the medium is decreased (Halestrap & Davidson, 1990). In Figure 9 we show that this is also the case in KCl medium. The rate of Ca-induced swelling was substantially greater when the KCl concentration of the medium was reduced from 150 to 75 mM which represents a decrease in osmolarity from 330 to 180 mosM. Under deenergized conditions in KCl medium this change in osmolarity increases the matrix volume of liver mitochondria from about 1.7 to 2.4 μL/mg of protein (Halestrap & Quinlan, 1983). The osmotically inactive space of mitochondria under these conditions is about 0.7 μL/mg and thus the increase in osmotically active matrix volume is about 70% (Halestrap & Quinlan, 1983). In parallel with the activation of Ca-induced swelling there is also a substantial increase in CyP binding as shown in the inset to Figure 9. In three separate experiments, the CyP binding in the hypoosmotic medium determined by scanning of blots such as that shown in Figure 9 was 179%, 226%, and 442% of the binding in isoosmotic medium. In the last two of these experiments the effect of 0.1 mM diamide was also studied to establish whether matrix volume could still exert an effect in the presence of diamide. Although an effect of hypoosmotic medium on CyP binding was still apparent in the presence of diamide (160% and 114%, respectively, of that observed in isoosmotic medium), the effect was less than that observed in the absence of this reagent.

DISCUSSION

Role of CyP Binding in Pore Opening. The present study and our previous work (Connern & Halestrap, 1994) have shown that thiol reagents, oxidative stress, increased matrix volume, decreased matrix NADH/NAD^+ ratio, and KSCN buffer all increase CyP binding to the inner mitochondrial membrane and enhance the sensitivity of pore opening to [Ca^{2+}]. Other factors that modulate the pore sensitivity to [Ca^{2+}] were without effect on CyP, including the addition of ADP, bongkrekate, and carboxyatractyloside (Connern & Halestrap, 1994) and membrane potential (Figure 4). We have also been unable to detect any decrease in CyP binding at pH 6.5 (data not shown), a pH at which pore opening is substantially inhibited (Halestrap, 1991; Bernardi *et al*.,

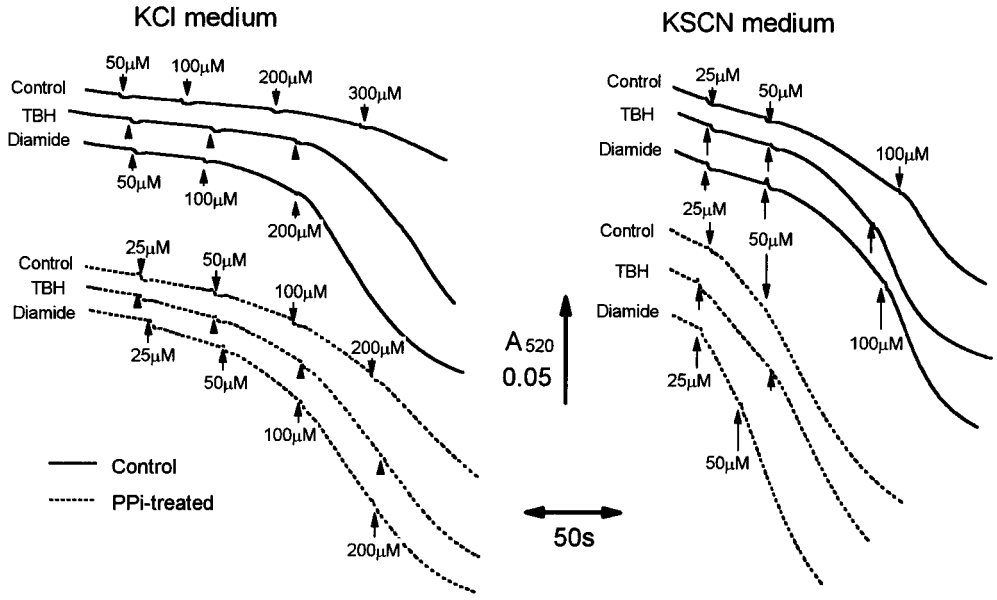

FIGURE 6: Comparison of Ca^{2+}-induced swelling of control and adenine nucleotide-depleted mitochondria in KCl and KSCN media. Swelling of deenergized mitochondria was measured in both KCl and KSCN media using the decrease in A_{520} induced by increasing concentrations of Ca^{2+} as described under Methods. Both control fresh mitochondria (solid lines) and those depleted of adenine nucleotides by PP_i treatment (dotted lines) were used. The $[Ca^{2+}]$ was buffered with NTA, and the values shown represent the free Ca^{2+} concentration. Where indicated, mitochondria were treated with 0.1 mM diamide or 1 mM TBH before the first calcium addition. Data for the maximal rates of swelling of the PP_i-treated mitochondria at varying $[Ca^{2+}]$ are shown in Figure 7.

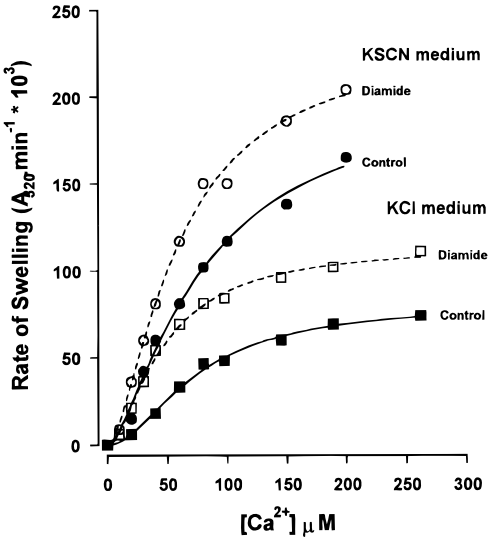

FIGURE 7: Dependence on $[Ca^{2+}]$ of the maximal rates of Ca^{2+}-induced swelling of adenine nucleotide-depleted mitochondria in KCl and KSCN media. The experimental protocol was as described for Figure 6. A separate mitochondrial incubation was used for each $[Ca^{2+}]$ and maximal rates of swelling after the addition of Ca^{2+} were determined by differentiation. Data are shown for PP_i-treated mitochondria with and without treatment for 1 min with 0.1 mM diamide before addition of Ca^{2+}.

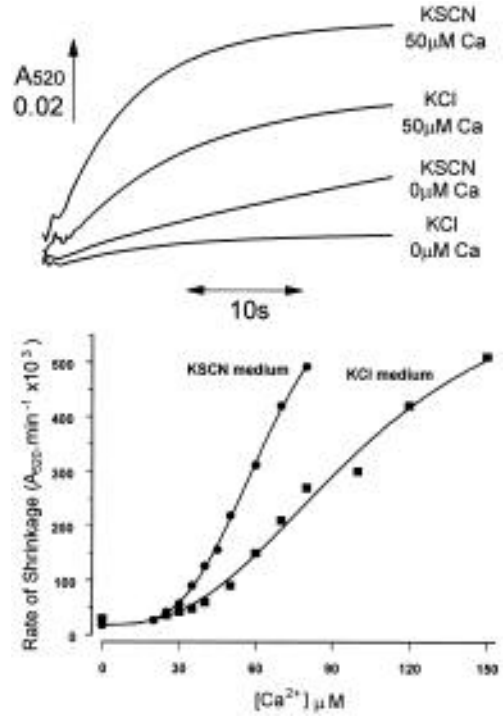

FIGURE 8: Dependence on $[Ca^{2+}]$ of the maximal rates of PEG-induced shrinking of preswollen mitochondria in KCl and KSCN media. Fresh mitochondria were preswollen in KSCN medium and then reequilibrated in KCl or KSCN medium as described under Methods. Shrinkage in KCl or KSCN medium containing the $[Ca^{2+}]$ shown was induced by the addition of 7% (w/v) PEG. Typical traces are shown in the top panel, while initial rates at increasing $[Ca^{2+}]$ are plotted in bottom panel.

1992). In contrast, Nicolli *et al.* (1996) have recently shown that the small amount of CyP remaining bound to submitochondrial particles (SMPs) in sucrose medium can be released by low pH (pH 5) as well as by CsA and isoosmotic NaCl. The effect of low pH was blocked by diethyl pyrocarbonate, which also prevents the inhibition of pore opening by low pH. However, it is unclear whether these observations reflect specific binding of CyP to its target protein rather than nonspecific binding of CyP to the membrane phospholipids, which act as an ion-exchange matrix. In our hands, CyP binding to SMPs in sucrose medium was nonspecific and many other matrix proteins (e.g., citrate synthase) also bind under these conditions. Indeed, Nicolli *et al.* (1996) also show the release of numerous different proteins from SMPs resuspended in sucrose medium at pH 5.

Our data are consistent with a model in which CyP binds to a target membrane protein that is a component of the pore

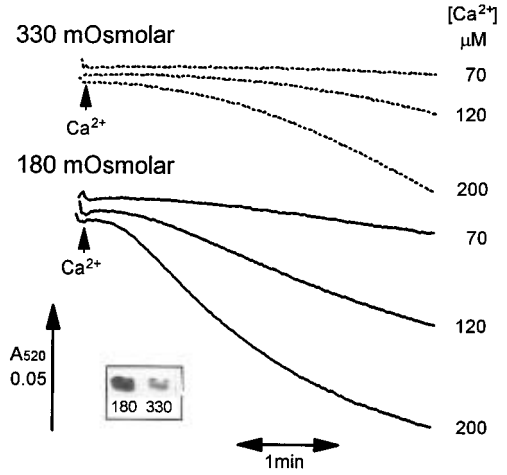

FIGURE 9: Sensitivity to $[Ca^{2+}]$ of Ca^{2+}-induced swelling of deenergized mitochondria in isoosmotic and hypoosmotic KCl media. The experimental protocol was as described for Figure 6, with the medium containing 20 mM Mops, 10 mM Tris, 2 mM NTA, 0.2 μM rotenone, 0.2 μM antimycin, 2 μM A23187, pH 7.2, and either 150 mM KCl (330 mosM) or 75 mM KCl (180 mosM) as indicated. Additions of $CaCl_2$ to give the required $[Ca^{2+}]$ were made at the times indicated. The inset shows CyP binding to membranes under the same conditions, measured as described in Figure 5.

structure and sensitizes it to opening at lower $[Ca^{2+}]$. However, other factors may exert their influence on the pore by binding directly to sites on the membrane protein. As we (Connern & Halestrap, 1994) and others (Bernardi & Petronilli, 1996) have noted previously, there are some parallels between this model and that proposed for the involvement of the FK506-binding protein (FKBP) in modulating Ca release mechanisms of the sarcoplasmic and endoplasmic reticulum. FK506-binding proteins are intimately associated with both the ryanodine receptor of the sarcoplasmic reticulum and the IP3 receptor of the endoplasmic reticulum. Their removal by addition of FK506 increases the probability of the channel being in the open state, leading to an increased sensitivity to caffeine or IP3 and a greater leakiness of the calcium stores (Timerman *et al*., 1993, 1994; Brillantes *et al*., 1994; Maryleitner *et al*., 1994; Cameron *et al*., 1995). Thus binding of CyP or FKBP to their target proteins could induce a conformation that is more sensitive to IP3 or caffeine. CyP and FKBP-related proteins are also complexed with and modulate the activities of steroid receptors (Smith *et al*., 1993a,b; Ratajczak *et al*., 1993; Kieffer *et al*., 1993), Type 1 receptors of the TGF-β family (Wang *et al*., 1994), and a 77-kDa surface glycoprotein of unknown function (Friedman *et al*., 1993).

In our previous model of pore opening we have assumed that CyP binding to its target membrane protein is essential for pore opening. If this were the case, then the more CyP that bound, the more pores that would open when triggered by Ca^{2+}. However, there is now a body of data to suggest that CyP binding may not be essential for pore opening but may merely sensitize the pore opening mechanism to $[Ca^{2+}]$. Thus studies on the megachannel of patched clamped mitochondria, which is thought to represent the pore, have shown that the inhibitory effect of CsA is overcome at higher $[Ca^{2+}]$ (Szabo & Zoratti, 1991; Zoratti & Szabo, 1994). Novgorodov *et al*. (1992) and Crompton and Andreeva (1994), using different techniques, have shown that at high matrix $[Ca^{2+}]$ inhibition of pore opening by CsA is overcome, and we have confirmed this observation in both heart mitochondria (Griffiths & Halestrap, 1995) and liver mitochondria (A. P. Halestrap, unpublished data) under conditions in which CsA greatly reduces the binding of CyP to the inner membrane (Connern & Halestrap, 1994). Thus the evidence favors a modulatory role of CyP on pore opening rather than an essential role. However we cannot exclude CyP binding being a prerequisite for pore opening, with the CsA−CyP complex fulfilling this role with a greatly reduced affinity.

Mechanism of Action of Chaotropic Agents. The ability of chaotropic agents such as thiocyanate and guanidinium to enhance CyP binding is of considerable interest and may give clues to the nature of the binding site. Effects of chaotropic agents on ligand/receptor and enzyme/substrate binding have been described in other systems. Low concentrations of KSCN (0−150 mM) or other chaotropic agents have been reported to enhance the activity of detergent-solubilized dihydroorotate dehydrogenase (Forman & Kennedy, 1977) and of chloroplast fructose-1,6-bisphosphatase (Stein & Wolosiuk, 1987). Binding of α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA), a ligand for the quisqualate/AMPA type of glutamate receptor, is increased in the presence of chaotropic ions such as thiocyanate (Maksay & Ticku, 1984; Honoré & Nielsen, 1985; Honoré & Drejer, 1988; Hall *et al*., 1992, 1993). Interestingly, thiol reagents also increase the affinity of the receptor for AMPA (Terramani *et al*., 1988), in an analogous fashion to their effect on mitochondrial CyP binding. Although the effect of thiocyanate on AMPA binding has been widely reported, the mechanism by which it exerts its effect is uncertain. Chaotropes stabilize hydrophobic groups in an aqueous environment and may favor a conformation of the protein in which hydrophobic residues involved in substrate/agonist binding are more exposed. A similar mechanism may apply for the binding of CyP to the inner mitochondrial membrane. If CyP binding to its target protein requires some hydrophobic interactions, and the residues of the target membrane protein involved in such interactions are more exposed in the presence of a chaotropic agent, then enhanced binding in the presence of chaotropes would be predicted. The effect of chaotropic agents is unlikely to be on the conformation of CyP itself, since we have been unable to detect any effect of 150 mM KSCN on the PPIase activity of mitochondrial CyP (data not shown).

The ability to stabilize the binding of CyP to its target membrane protein by the addition of chaotropic agents may provide a means by which the target protein can be identified. Thus, if the complex were to remain stable during detergent solubilization, then chromatographic techniques might be able to separate bound and free cyclophilin and so purify the binding partner. However, using diamide-treated mitochondria solubilized with either Triton X-100 or dodecyl maltoside in the presence or absence of thiocyanate, we have been unable to detect such a cyclophilin/protein complex in any fraction following gel-filtration chromatography.

Is There a Mitochondrial CyP Integral to the Inner Membrane and Distinct from the Matrix Isoform? The emphasis of this paper has been on the mechanisms involved in the recruitment of matrix CyP to the putative target protein of the pore, and its probable role in modulating pore activity. However, recently Crompton and colleagues have reported the presence of a 22-kDa CyP associated with the inner membrane of heart mitochondria (Andreeva *et al*., 1995).

This CyP was found to bind a photoreactive form of CsA, binding being greater in the presence of ADP and less in the presence of Ca^{2+}, which inhibit and activate pore opening respectively (Crompton & Andreeva, 1994; Andreeva *et al*., 1995). This has led Crompton and colleagues to propose that it is this distinct membrane-bound cyclophilin that is critical for regulating the pore, rather than the matrix isoform. In our Percoll-purified heart and liver mitochondria we have been unable to detect any such 22-kDa form of CyP with our N-terminal anti-peptide antibody, although in the cytosol a 22-kDa form can be detected and probably represents the precursor protein of mitochondrial CyP, whose sequence has been reported by Bergsma *et al*. (1991). Although, like Crompton and colleagues, we have also been able to detect very low PPIase activity in our purified membrane preparations, parallel measurements of citrate synthase activity confirms that this can all be accounted for by matrix contamination (Connern & Halestrap, 1994). We are thus uncertain as to the identity of the 22-kDa CyP of Crompton and colleagues. Although cloning studies provide no evidence for a new 22-kDa CyP isoform (Bergsma *et al*., 1991), we have investigated whether there could be an additional isoform of CyP against which our antibody is inactive by synthesizing an anti-peptide antibody against an internal sequence (ANAGPNTN) conserved in most 18−22-kDa CyPs. However, this also failed to detect any 22-kDa mitochondrial CyP despite detecting CyP-A and ER-associated CyPs (data not shown). Nicolli *et al*. (1996) have also been unable to detect any mitochondrial CyP other than CyP-D. The endoplasmic reticulum contains an isoform of 20 kDa (Bose & Freedman, 1994; Bose *et al*., 1994) and it is of note that on SDS−PAGE, the cyclophilin identified by Andreeva *et al*. (1995) had the same mobility as the trypsin inhibitor protein (20.1 kDa). Thus it is likely that the 22-kDa CyP detected by Crompton and colleagues is due to sarcoplasmic reticulum contamination of the mitochondrial fraction.

REFERENCES

Andreeva, L., & Crompton, M. (1994) *Eur. J. Biochem. 221*, 261−268.

Andreeva, L., Tanveer, A., & Crompton, M. (1995) *Eur. J. Biochem. 230*, 1125−1132.

Bergsma, D. J., Eder, C., Gross, M., Kersten, H., Sylvester, D., Appelbaum, E., Cusimano, D., Livi, G. P., Mclaughlin, M. M., Kasyan, K., Porter, T. G., Silverman, C., Dunnington, D., Hand, A., Prichett, W. P., Bossard, M. J., Brandt, M., & Levy, M. A. (1991) *J. Biol. Chem. 266*, 23204−23214.

Bernardi, P. (1992) *J. Biol. Chem. 267*, 8834−8839.

Bernardi, P., & Petronilli, V. (1996) *J. Bioenerg. Biomembr. 28*, 131−138.

Bernardi, P., Broekemeier, K. M., & Pfeiffer, D. R. (1994) *J. Bioenerg. Biomembr. 26*, 509−517.

Bose, S., & Freedman, R. B. (1994) *Biochem. J. 300*, 865−870.

Bose, S., Mucke, M., & Freedman, R. B. (1994) *Biochem. J. 300*, 871−875.

Broekemeier, K. M., Dempsey, M. E., & Pfeiffer, D. R. (1989) *J. Biol. Chem. 264*, 7826−7830.

Cameron, A. M., Steiner, J. P., Sabatini, D. M., Kaplin, A. I., Walensky, L. D., & Snyder, S. H. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1784−1788.

Chernyak, B. V., Dedov, V. N., & Chernyak, V. Y. (1995) *FEBS Lett. 365*, 75−78.

Colley, N. J., Baker, E. K., Stamnes, M. A., & Zuker, C. S. (1991) *Cell 67*, 255−263.

Connern, C. P., & Halestrap, A. P. (1992) *Biochem. J. 284*, 381−385.

Connern, C. P., & Halestrap, A. P. (1994) *Biochem. J. 302*, 321−324.

Crompton, M. (1990) in *Calcium and the Heart* (Langer, G. A., Ed.) pp 167−198, Raven Press Ltd., New York.

Crompton, M., & Andreeva, L. (1994) *Biochem. J. 302*, 181−185.

Crompton, M., Costi, A., & Hayat, L. (1987) *Biochem. J. 245*, 915−918.

Crompton, M., Ellinger, H., & Costi, A. (1988) *Biochem. J. 255*, 357−360.

Diggle, T. A., Bloomberg, G. B., & Denton, R. M. (1995) *Biochem. J. 306*, 135−139.

Forman, H. J., & Kennedy, J. (1977) *J. Biol. Chem. 252*, 3379−3381.

Friedman, J., Trahey, M., & Weissman, I. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6815−6819.

Fruman, D. A., Burakoff, S. J., & Bierer, B. E. (1994) *FASEB J. 8*, 391−400.

Galat, A., & Metcalfe, S. M. (1995) *Prog. Biophys. Mol. Biol. 63*, 67−118.

Griffiths, E. J., & Halestrap, A. P. (1991) *Biochem. J. 274*, 611−614.

Griffiths, E. J., & Halestrap, A. P. (1993) *J. Mol. Cell. Cardiol. 25*, 1461−1469.

Griffiths, E. J., & Halestrap, A. P. (1995) *Biochem. J. 307*, 93−98.

Gunter, T. E., & Pfeiffer, D. R. (1990) *Am. J. Physiol. 258*, C755−C786.

Halestrap, A. P. (1991) *Biochem. J. 278*, 715−719.

Halestrap, A. P. (1994) in *Mitochondria: DNA, proteins and disease* (Darley-Usmar, V., & Schapira, A. H. V., Eds.) pp 113−142, Portland Press Ltd., Colchester, Essex, U.K.

Halestrap, A. P., & Quinlan, P. T. (1983) *Biochem. J. 214*, 387−393.

Halestrap, A. P., & Davidson, A. M. (1990) *Biochem. J. 268*, 153−160.

Hall, R. A., Kessler, M., & Lynch, G. (1992) *J. Neurochem. 59*, 1997−2004.

Hall, R. A., Massicotte, G., Kessler, M., Baudry, M., & Lynch, G. (1993) *Mol. Pharmacol. 43*, 459−464.

Hatefi, Y., & Hanstein, W. G. (1973) *Methods Enzymol. 31*, 770−790.

Helekar, S. A., Char, D., Neff, S., & Patrick, J. (1994) *Neuron 12*, 179−189.

Honoré, T., & Nielsen, M. (1985) *Neurosci. Lett. 54*, 27−32.

Honoré, T., & Drejer, J. (1988) *J. Neurochem. 51*, 457−461.

Kieffer, L. J., Thalhammer, T., & Handschumacher, R. E. (1992) *J. Biol. Chem. 267*, 5503−5507.

Klingenberg, M. (1976) in *The Enzymes of Biological Membranes* (Martonosi, A., Ed.) Vol. 3, pp 383−483, Plenum, New York.

Lodish, H. F., & Kong, N. (1991) *J. Biol. Chem. 266*, 14835−14838.

Maksay, G., & Ticku, M. K. (1984) *J. Neurochem. 43*, 261−268.

Maryleitner, M., Timerman, A. P., Wiederrecht, G., & Fleischer, S. (1994) *Cell Calcium 15*, 99−108.

McClaughlin, S., Bruder, A., Chen, S., & Moser, C. (1975) *Biochim. Biophys. Acta 394*, 304−313.

McCormack, J. G., Halestrap, A. P., & Denton, R. M. (1990) *Physiol. Rev. 70*, 391−425.

Nicolli, A., Basso, E., Petronilli, V., Wenger, R. M., & Bernardi, P. (1996) *J. Biol. Chem. 271*, 2185−2212.

Novgorodov, S. A., Gudz, T. I., Milgrom, Y. M., & Brierley, G. P. (1992) *J. Biol. Chem. 267*, 16274−16282.

Petronilli, V., Cola, C., & Bernardi, P. (1993a) *J. Biol. Chem. 268*, 1011−1016.

Petronilli, V., Cola, C., Massari, S., Colonna, R., & Bernardi, P. (1993b) *J. Biol. Chem. 268*, 21939−21945.

Petronilli, V., Costantini, P., Scorrano, L., Colonna, R., Passamonti, S., & Bernardi, P. (1994a) *J. Biol. Chem. 269*, 16638−16642.

Petronilli, V., Nicolli, A., Costantini, P., Colonna, R., & Bernardi, P. (1994b) *Biochim. Biophys. Acta 1187*, 255−259.

Rassow, J., Mohrs, K., Koidl, S., Barthelmess, I. B., Pfanner, N., & Tropschug, M. (1995) *Mol. Cell. Biol. 15*, 2654−2662.

Ratajczak, T., Carrello, A., Mark, P. J., Warner, B. J., Simpson, R. J., Moritz, R. L., & House, A. K. (1993) *J. Biol. Chem. 268*, 13187−13192.

Rigobello, M. P., Turcato, F., & Bindoli, A. (1995) *Arch. Biochem. Biophys. 319*, 225−230.

Schmid, F. X. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 123−143.

Schneider, H., Charara, N., Schmitz, R., Wehrli, S., Mikol, V., Zurini, M. G. M., Quesniaux, V. F. J., & Movva, N. R. (1994) *Biochemistry 33*, 8218−8224.

Schreiber, S. L., & Crabtree, G. R. (1992) *Immunol. Today 13*, 136−142.

Smith, D. F., Baggenstoss, B. A., Marion, T. N., & Rimerman, R. A. (1993a) *J. Biol. Chem. 268*, 18365−18371.

Smith, D. F., Albers, M. W., Schreiber, S. L., Leach, K. L., & Deibel, M. R. (1993b) *J. Biol. Chem. 268*, 24270−24273.

Stein, M., & Wolosiuk, R. A. (1987) *J. Biol. Chem. 262*, 16171−16179.

Steinmann, B., Bruckner, P., & Supertifurga, A. (1991) *J. Biol. Chem. 266*, 1299−1303.

Szabo, I., & Zoratti, M. (1991) *J. Biol. Chem. 266*, 3376−3379.

Terramani, T., Kessler, M., Lynch, G., & Baudry, H. (1988) *Mol. Pharamacol. 34*, 117−123.

Timerman, A. P., Ogunbumni, E., Freund, E., Wiederrecht, G., Marks, A. R., & Fleischer, S. (1993) *J. Biol. Chem. 268*, 22992−22999.

Timerman, A. P., Jayaraman, T., Wiederrecht, G., Onoue, H., Marks, A. R., & Fleischer, S. (1994) *Biochem. Biophys. Res. Commun. 198*, 701−706.

Wang, T. W., Donahoe, P. K., & Zervos, A. S. (1994) *Science 265*, 674−676.

Zoratti, M., & Szabo, I. (1994) *J. Bioenerg. Biomembr. 26*, 543−553.

BI9525177