THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 51, Issue of December 21, pp. 48027–48030, 2001
Printed in U.S.A.

Mitochondrial Creatine Kinase and Mitochondrial Outer Membrane Porin Show a Direct Interaction That Is Modulated by Calcium*

Received for publication, July 12, 2001, and in revised form, September 28, 2001
Published, JBC Papers in Press, October 15, 2001, DOI 10.1074/jbc.M106524200

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Mitochondrial creatine kinase (MtCK) co-localizes with mitochondrial porin (voltage-dependent anion channel) and adenine nucleotide translocator in mitochondrial contact sites. A specific, direct protein-protein interaction between MtCK and mitochondrial porin was demonstrated using surface plasmon resonance spectroscopy. This interaction was independent of the immobilized binding partner (porin reconstituted in liposomes or MtCK) or the analyzed isoform (chicken sarcomeric MtCK or human ubiquitous MtCK, human recombinant porin, or purified bovine porin). Increased ionic strength reduced the binding of MtCK to porin, suggesting predominantly ionic interactions. By contrast, micromolar concentrations of Ca^{2+} increased the amount of bound MtCK, indicating a physiological regulation of complex formation. No interaction of MtCK with reconstituted adenine nucleotide translocator was detectable in our experimental setup. The relevance of these findings for structure and function of mitochondrial contact sites is discussed.

The mitochondrial isoenzymes of creatine kinase (MtCK),[1] sarcomeric sMtCK, and ubiquitous uMtCK are strictly localized within the cristae and the intermembrane space of mitochondria where they convert mitochondrially produced ATP into phosphocreatine, another "high-energy" compound. MtCK, as a peripheral membrane protein, binds to the outer leaflet of the entire inner mitochondrial membrane and is specifically enriched in the so-called contact sites where inner and outer membrane are in close proximity. Here MtCK forms a functional microcompartment together with the transmembrane proteins adenine nucleotide translocator (ANT) in the inner membrane and mitochondrial porin (voltage-dependent anion channel) in the outer membrane (for reviews, see Refs. 1–3). The three proteins maintain a privileged exchange of MtCK substrates and products called functional coupling or substrate channeling (4, 5). Contact sites are therefore considered to play an important role in dynamic compartmentation of adenine nucleotides in the intermembrane space (6) and in high-energy phosphate channeling. It is also suggested that contact site complexes can constitute a functional and structural element of the permeability transition pore. The opening of the permeability transition pore, possibly regulated by MtCK oligomers (7), is considered a key event in the mitochondrial pathway leading to cellular apoptosis (8).

Despite the multiple functional interactions of contact site components, their exact topology and putative structural interactions have remained elusive. Chemical cross-linking of contact site proteins has failed so far (9, 10). Co-purification of MtCK, ANT, and porin in proteolipid contact site complexes only indicated a close proximity of these proteins (11). Finally, octamerization of a partially octamerization-incompetent MtCK mutant by addition of porin suggested that porin interacts with MtCK at some stage of octamer formation (12, 13). However, no unambiguous proof for a permanent direct protein-protein interaction between these two proteins has been reported yet.

The recent introduction of surface plasmon resonance (SPR) spectroscopy for the analysis of MtCK binding to membranes (14–16) has opened the avenue for a detailed evaluation of the postulated MtCK/porin interaction. Here, by using BIAcore SPR technology, we compare binding of MtCK to pure phosphatidylcholine vesicles with its binding to vesicles containing reconstituted mitochondrial porin. We can show a specific MtCK/porin interaction that depends on porin concentration, involves ionic interactions, and is modulated by calcium.

EXPERIMENTAL PROCEDURES

Proteins—Chicken sMtCK and human uMtCK were expressed in *Escherichia coli* and purified to homogeneity according to Furter *et al.* (17) and Schlattner *et al.* (18). Purified proteins were stored at −80 °C at 3–6 mg/ml in 50 mM NaP_i at pH 7.0, 150 mM NaCl, 0.2 mM EDTA, and 2 mM β-mercaptoethanol. Before use, dilutions were made in 10 mM TES, pH 7.0, and 50 mM NaCl. Recombinant human mitochondrial porin was expressed in *E. coli*, purified as described earlier (19), and stored at a concentration of 6–10 mg/ml at 4 °C in 10 mM TES, pH 7.0, 50 mM NaCl, 1% octylpolyoxyethylene, and 10% glycerol. Bovine porin was purified to homogeneity from beef heart mitochondria. In short, 40 mg of mitochondria were solubilized on ice with 4 ml of 150 mM Na_2SO_4, 50 mM HEPES, pH 7.0, 1 mM EDTA, 3% CHAPS (buffer A), vortexed, and centrifuged in an air-driven ultracentrifuge (Beckman Instruments) at 30 p.s.i. for 5 min. The clear supernatant was loaded on a column containing 2 g of dry hydroxyapatite, and proteins were eluted with buffer A. The first 4 ml of the eluate were diluted with 20 mM MES, pH 6.0, 0.2 mM EDTA, and 1% CHAPS (buffer B) in a ratio of 1:3 (v/v) and loaded on a 1-ml cation exchange column (HiTrap SP, Amersham Biosciences AB, Uppsala, Sweden). Under these conditions, porin does not bind to the column matrix and was collected in the flow-through, whereas contaminating proteins (mainly ANT) were eluted with 1 M NaCl in buffer B. Porin-containing fractions were concentrated to 0.17–0.35 mg/ml using Centriprep-30 devices (Amicon Inc., Beverly, MA,) checked for purity by SDS-PAGE, and used for reconstitution into phosphatidylcholine (PC) vesicles.

Other Chemicals—Egg yolk PC was from Lipid Products (South Nutfield, United Kingdom), hydroxyapatite was from Bio-Rad, avidin

* This work was supported by grants from the Sandoz Family Office (to M. T.-S. and U. S.), Novartis Stiftung, Schweizerische Herzstiftung, and Wolfermann-Nägeli Stiftung (to T. W. and M. T.-S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MtCK, mitochondrial creatine kinase; ANT, adenine nucleotide translocator; PC, phosphatidylcholine; sMtCK, sarcomeric MtCK; SPR, surface plasmon resonance; uMtCK, ubiquitous MtCK; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; MES, 4-morpholineethanesulfonic acid; RU, resonance unit(s).

and cardiolipin were from Sigma, and all other chemicals were from Fluka (Buchs, Switzerland).

Reconstitution of Porin and Preparation of Liposomes—A lipid stock suspension at 5 mg/ml containing PC (99.9% w/w) and biotin-X-DHPE (*N*-((6-biotinoyl)amino)hexanoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine) (0.1% w/w) in 10 mM TES, pH 7.0, and 50 mM NaCl was prepared as described by Schlattner and Wallimann (16). Porin was reconstituted according to Bathori *et al.* (20) with minor modifications. Briefly, the PC suspension and porin preparation were mixed at lipid:protein ratios of 10:1, 4:1, or 2:1 (w/w). Equivalent amounts of porin storage buffer were added to the control PC suspension. The mixtures were vortexed, sonicated, and dialyzed for 40 h against 10 mM TES, pH 7.0, and 50 mM NaCl at 4 °C to remove detergent. Liposomes were produced by a combination of freeze/thawing and extrusion techniques (16), stored at 4 °C, and used within 2 days. The quality and reproducibility of preparations were checked by transmission electron microscopy.

Surface Plasmon Resonance (SPR) Analysis—Binding studies were done using surface plasmon resonance spectroscopy with a BIAcore 2000™ (Biacore, Uppsala, Sweden) according to Schlattner and Wallimann (15, 16). Binding is expressed in arbitrary resonance units (RU). Interaction between MtCK and PC vesicles with or without reconstituted porin was determined in two different setups: setup A, biotinylated lipid vesicles were immobilized for each measurement on an avidin-coated CM5 sensorchip, and MtCK was injected in the flow; or setup B, MtCK was covalently immobilized on a CM5 sensorchip using routine amine coupling, and lipid vesicles were injected. Programmed measurement cycles (immobilization of liposomes in setup A, contact phase and dissociation phase of 170–240 s each, regeneration with 1% SDS) were performed at 25 °C and a flow rate of 0.3 ml h^{-1} in running buffer (10 mM TES, pH 7.0, 50 mM NaCl) if not stated otherwise. Additional experiments in setup B were performed with vesicles preincubated in TES buffer supplemented with 3.0 mM $MgCl_2$, 0.1 mM EGTA, and $CaCl_2$ to obtain 10 μM free calcium. Control and porin-containing samples were injected in random order. Each cycle was repeated at least once to demonstrate that binding responses were reproducible. SPR data were corrected for background binding and refractive index changes using the signal from reference lanes either coated with avidin (setup A) or a blank lane (setup B).

Extraction of Lipids—Lipids that co-purified with porin from beef mitochondria were isolated by precipitating protein and extracting lipids with chloroform according to De Pinto *et al.* (21). The lipid fraction remaining after evaporation of chloroform was resuspended in porin storage buffer (1:3 (v/v) mixture of buffers A and B) to obtain the initial volume. This preparation was incorporated into PC vesicles in the same way as the bovine porin preparation and used in control experiments to assess binding of MtCK to phospholipids that were co-purified with bovine porin.

RESULTS AND DISCUSSION

Purified Porin Can Be Reconstituted into Liposomes at High Density—The high quality and the reproducibility of the liposome preparations were confirmed by electron microscopy (Fig. 1). Pure PC vesicles and vesicles with reconstituted porin were very similar in size with diameters of 100–400 nm when observed by negative stain electron microscopy. Porin-containing vesicles were less smooth than pure PC vesicles, indicating the presence of reconstituted protein. Higher magnification revealed porin particles at very high density (Fig. 1*d*). Human recombinant porin at higher protein:lipid ratios protein was sometimes present as crystalline patches (see Ref. 19).

MtCK Directly Interacts with Mitochondrial Porin but Not with ANT—Interaction of MtCK with porin was analyzed by surface plasmon resonance with two different experimental setups: either using immobilized liposomes for analysis of MtCK binding (Fig. 2) or an inverse system with immobilized MtCK to analyze liposome binding (Fig. 3). We also applied different MtCK isoenzymes (human uMtCK or chicken sMtCK, both recombinant) as well as different porin preparations (recombinant human porin or purified bovine porin). Under all experimental conditions, the interaction between MtCK and porin-containing PC vesicles was significantly above the background binding to pure PC vesicles.

In parallel experiments with reconstituted ANT purified from beef heart mitochondria, we could not detect any signal above background, indicating the absence of a direct MtCK/ANT interaction under our experimental conditions (data not shown). These results confirm an earlier study that was unable to detect an interaction between MtCK and reconstituted ANT (22). In addition, both MtCK and ANT have a basic pI, which makes a direct interaction rather unlikely (23). *In vivo* an interaction between these two proteins may be mediated by cardiolipin patches surrounding ANT. However, the amount of co-purified cardiolipin in our preparations was probably too small to allow such an interaction.

MtCK Binding Depends on Porin Concentration but Not on the Experimental Setup—The binding of chicken sMtCK or human uMtCK (Fig. 2) to immobilized PC vesicles containing different amount of bovine porin showed typical saturation kinetics. End association and end dissociation responses depended on porin concentration as seen with the SPR data corrected for background binding to PC (Fig. 2*b*). The very slow

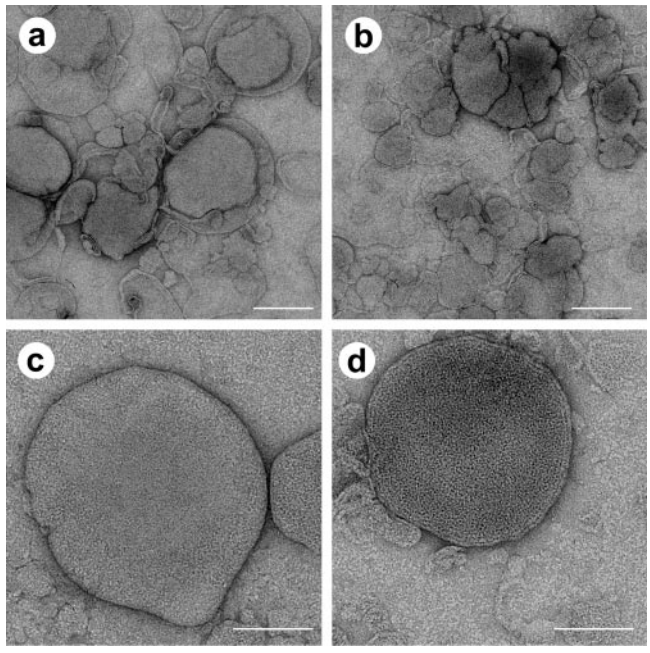

FIG. 1. **Electron micrographs of lipid vesicles.** Pure PC vesicles (*a* and *c*) and PC vesicles with reconstituted recombinant human porin at a lipid:protein ratio of 10:1 (*b* and *d*). *a* and *b*, general overview; *c* and *d*, high-magnification view showing a single vesicle. Lipid concentrations of the preparations were 25 μg/ml. *Bars* correspond to 0.2 μm (*a* and *b*) and 0.1 μm (*c* and *d*). Samples were stained with 1% uranyl acetate.

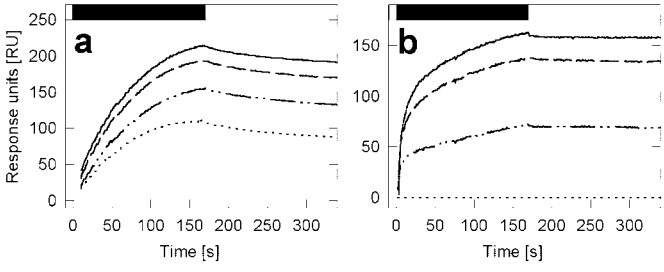

FIG. 2. **Interaction of MtCK with immobilized PC vesicles containing reconstituted porin isolated from beef heart mitochondria.** Two representative sets of SPR kinetics showing contact phase (*black bar*) and dissociation phase (*white bar*) are shown. *a*, data set corrected for background binding; *b*, data set corrected for nonspecific binding to PC. Pure PC vesicles (·····) and vesicles containing porin at lipid:protein ratios (w/w) of 10:1 (–·–·–), 4:1 (– – –), and 2:1 (——) are shown. Human uMtCK at 50 μg/ml was injected in the running buffer (10 mM TES, pH 7.0, 50 mM NaCl) onto immobilized vesicles (1300 RU).

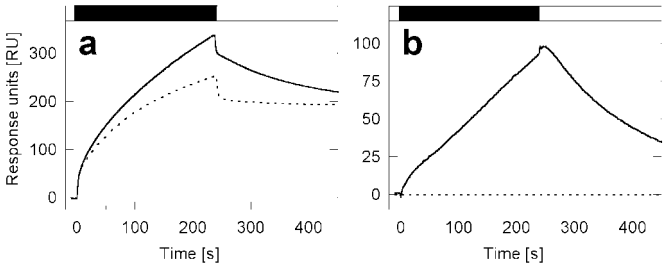

FIG. 3. **Interaction of PC vesicles containing reconstituted human recombinant porin with MtCK.** Representative SPR traces of contact phase (*black bar*) and dissociation phase (*white bar*) are shown. *a*, data corrected for background binding; *b*, data corrected for nonspecific binding of PC vesicles. Pure PC vesicles (·····) and vesicles containing porin at lipid:protein ratios (w/w) of 10:1 (——) are shown. Vesicles at 500 μg/ml were injected in the running buffer (10 mM TES, pH 7.0, 50 mM NaCl) onto immobilized chicken sMtCK (23000 RU).

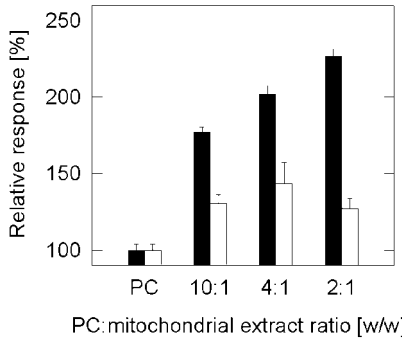

FIG. 4. **Contribution of lipids co-purified with porin from beef heart mitochondria on MtCK interaction.** The SPR response was measured for chicken sMtCK at 50 μg/ml injected in the running buffer (10 mM TES, pH 7.0, 50 mM NaCl) onto immobilized PC vesicles (1000 RU), and response values were taken at 120 s of dissociation phase. The vesicles contained either only PC or were supplemented with increasing amounts of a porin preparation from beef mitochondria (*black bars*) or corresponding volumes of a protein-free lipid extract from the same porin preparation (*white bars*). Response values were normalized in relation to the response with pure PC vesicles which was taken as 100%. Values are given as mean ± S.E. (n = 3).

dissociation of the MtCK·porin complex indicated a rather high affinity. However, because of this kinetic stability of the complex, reliable k_{off} values cannot be calculated from the SPR kinetics. Since this would be necessary to verify the consistency of parameters derived from SPR data (24), we were reluctant to calculate exact kinetic and equilibrium constants. Calculations based on the association kinetics alone yielded affinity values more than 1 order of magnitude lower than those for the MtCK/cardiolipin interaction, which are about 16–86 nM, depending on the isoenzyme (15).

We verified the MtCK/porin interaction in an inverse system with immobilized MtCK and recombinant human porin (Fig. 3). Again the presence of reconstituted porin increased the binding of vesicles to MtCK, albeit the binding kinetics was quite different from that in the setup with immobilized vesicles. The linear character of association kinetics and the pronounced dissociation as compared with the first setup (Fig. 3*b*) indicate a more complex binding mechanism, which is most probably due to a dissociation of the covalently immobilized MtCK octamers into dimers. Dimeric MtCK displays weak binding with quasilinear association kinetics to cardiolipin-containing PC vesicles as shown in an earlier study (see Fig. 3 in Ref. 16). In addition, the SPR signal in this setup may be influenced by coalescent vesicles, changes in vesicle shape, or detachment of the very bulky liposomes from the surface by shearing forces of the flow. Porin-containing vesicles, in contrast to pure PC vesicles, also interacted with immobilized cytochrome *c* and hexokinase (data not shown). This is in accordance with published data (25, 26) and further supports the reliability of our experimental system.

Co-purified Lipids in Porin Preparations Are Not Responsible for MtCK Binding—Porin preparations from beef heart mitochondria contained remnants of lipids, mainly PC, phosphatidylethanolamine, and cholesterol, but also smaller amounts of cardiolipin as shown by thin layer chromatography (data not shown). The presence of cardiolipin is critical since it is the high affinity receptor of MtCK in the inner mitochondrial membrane (22, 27) and it might contribute to the observed interaction of MtCK with porin-containing vesicles. Therefore, we compared MtCK binding to immobilized vesicles in either the full porin preparation or the extracted protein-free lipid fraction (Fig. 4). Although the presence of the lipid fraction increased MtCK binding to some extent, binding was much weaker and did not show concentration dependence as in the case of porin-containing vesicles. This clearly confirms that interaction mainly occurs between MtCK and porin.

Binding Partners and Binding Mode of MtCK in Mitochondria—Cardiolipin and other phospholipids with negative net charge have been identified as binding partners of MtCK (22, 27), and a detailed quantitative characterization of these interactions is available (14–16, 28). Other binding partners have been ruled out so far on the basis of cross-linking studies (9, 10). Now, in addition to phospholipids, porin can be classed as a new binding partner for MtCK in the outer mitochondrial membrane. Exact quantitation of kinetic constants was not possible, but the kinetic stability of the MtCK·porin complex indicates a rather high affinity (Fig. 2). By contrast, porin vesicles seem to offer much less binding sites per surface unit as compared with cardiolipin vesicles. With immobilized vesicles, the maximal equilibrium response of 0.15 μM human uMtCK is about 900 RU for 16% cardiolipin vesicles (500 RU immobilized; Ref. 15), while it is less than 100 RU for vesicles with comparably low porin content (*i.e.* lipid:protein ratio (w/w) of 10:1, 1300 RU immobilized; see Fig. 2*b*). Possibly not every reconstituted porin molecule can contribute to binding of MtCK due to wrong orientation in the membrane or misfolding. Buffers with higher ionic strength (100 mM salt) reduced binding of MtCK to porin vesicles (data not shown). This suggests the participation of electrostatic forces similar to the known MtCK/cardiolipin interaction rather than the presence of hydrophobic interactions postulated earlier (12). A candidate for the MtCK-binding domain of porin would be the flexible N terminus, which is known to interact with cytochrome *c* (26), a protein very similar to MtCK in its basic pI and its high affinity to cardiolipin.

Calcium Increases MtCK/Porin Interaction—Of several effectors examined, micromolar concentrations of calcium (10 μM) in the presence of magnesium (0.3 mM) increased binding of porin-containing vesicles to immobilized human uMtCK (Fig. 5). The same concentrations of Ca^{2+} and Mg^{2+} had little or no influence on the SPR signal when porin-containing vesicles were injected onto immobilized cytochrome *c* or bovine serum albumin (data not shown). This finding could be physiologically relevant if contact site formation is considered a dynamic process. Raising intracellular free calcium levels, *e.g.* upon activation of muscle contraction, would trigger the formation and/or stabilization of MtCK·porin complexes. In fact, a study in perfused heart suggested a link between calcium concentration and contact site formation (29).

Significance of MtCK/Porin Interaction in Mitochondrial Contact Sites—The structural basis for the substrate channeling between ANT, MtCK, and porin in peripheral contact sites of mitochondria has long remained enigmatic. A major diffi-

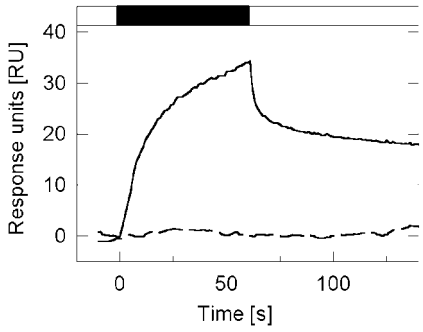

FIG. 5. **Influence of calcium on porin/MtCK interaction.** SPR difference traces representing the additional SPR response in the presence of 10 μM Ca^{2+} as compared with Ca^{2+}-free buffer (see "Experimental Procedures"), contact phase (*black bar*), and dissociation phase (*white bar*). Measurements were done using pure PC vesicles (– – –) or PC vesicles containing human recombinant porin at lipid:protein ratios (w/w) of 10:1 (——). Vesicles at 25 μg/ml were injected in the running buffer (10 mM TES, pH 7.0, 50 mM NaCl) onto immobilized chicken sMtCK (25,000 RU).

culty has been to explain the interaction of the highly symmetrical, cuboidal MtCK octamer with the outer and inner membranes, which differ substantially in their properties. While MtCK has two identical four-fold symmetry faces that interact with membranes (30), the two mitochondrial membranes differ entirely in their lipid and protein composition. The inner membrane contains high amounts of cardiolipin that will strongly interact with positively charged amino acid residues at the C terminus of MtCK.[2] Since cardiolipin also strongly binds to ANT (31, 32), the existence of cardiolipin patches in the inner membrane would be sufficient to bring ANT and MtCK in close vicinity as postulated for functional coupling (Ref. 33 and reviewed in Refs. 3 and 34) and to allow co-purification of ANT·MtCK·porin complexes (11). For interaction of MtCK with porin in the outer membrane, which is almost deficient of cardiolipin, a similar indirect complex formation via other charged phospholipids has been proposed (9). However, our present data clearly show that, in contrast to ANT, porin can directly interact with MtCK. This confirms previous indirect evidence for *in vitro* complex formation between MtCK and porin (12).

Our finding on porin as a MtCK receptor in the outer membrane is not only important for contact site topology and metabolic channeling therein (for a review, see Ref. 2) but seems also to be relevant for the potential implication of MtCK in apoptosis-related functions of contact sites. ANT and voltage-dependent anion channel were proposed to trigger apoptosis by interacting with proapoptotic Bcl-2 family members (35, 36) and/or forming the mitochondrial permeability transition pore (for reviews, see Refs. 8 and 37) as indicated by studies with reconstituted contact site complexes (11). MtCK may interfere with these processes as permeability transition pore opening was shown to be inhibited by octameric MtCK together with its substrates (7).

[2] U. Schlattner, F. Gehring, and T. Wallimann, unpublished data.

Finally, our study extends the application of real-time SPR technology to the field of interactions between peripheral membrane proteins with reconstituted integral membrane proteins. This provides a very attractive tool for interaction proteomics, which will be especially important for the molecular analysis of metabolic channeling and cellular signal transduction.

Acknowledgment—We thank Magda Livingstone for reading the manuscript.

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J. Biol. Chem. 2001, 276:48027-48030.
originally published online December 14, 2001
