Function of M-line-bound Creatine Kinase As Intramyofibrillar ATP Regenerator at the Receiving End of the Phosphorylcreatine Shuttle in Muscle

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After 10 wash cycles, 0.8 u.e. of creatine kinase activity remained bound per mg of chicken pectoralis myofibrils which had been freed of soluble creatine kinase, mitochondria, and membranes. The bound creatine kinase is located at the M-band and contributes to the electron density of this sarcemeric structure (Wallimann, T., Pelloni, G. W., Turner, D. C., and Eppenberger, H. M. 1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4296–4300. By measuring the combined actin-activated Mg2+-ATPase and creatine kinase reactions of myofibrils by pH-stat, it was shown that the amount of M-line-bound creatine kinase activity was sufficient to rephosphorylate the ATP hydrolyzed in vitro by the actin-activated Mg2+-ATPase. The amount of M-line-bound creatine kinase and thus the ATP regeneration potential depended on the muscle type. It was higher in fast muscles and lower in slow muscles. Inhibition of myofibrillar creatine kinase or extraction of the M-line-bound enzyme abolished the ATP regeneration potential without affecting ATPase activity. Inhibitors of myokinase, mitochondrial ADP/ATP translocase, and respiration did not affect the ATP regeneration potential or the ATPase. M-line-bound creatine kinase, sufficient to support an ATP turnover rate of 6 s⁻¹ per myosin head, seems to have the capacity for the intramyofibrillar regeneration of most or all of the ATP hydrolyzed by the myofibrillar ATPase during muscle contraction. Thus, M-line-bound creatine kinase at the myofibrillar receiving end of the phosphorylcreatine shuttle is of physiological significance.

Upon activation of muscle, phosphorylcreatine is efficiently transphosphorylated by creatine kinase (EC 2.7.3.2.) to yield ATP as the immediate source of energy for muscle contraction. Creatine kinase is involved in maintaining proper intracellular ATP/ADP ratios and phosphorylcreatine pool sizes and is therefore a key enzyme in muscle energetics (for review see Ref. 2).

A small but significant amount of MM-creatine kinase, at least 5% of the total creatine kinase activity present in skeletal muscle, is located within the myofibrillar apparatus at the M-band of the sarcemere (3–8). Specific anti-M-creatine kinase antibodies stain the M-band and render this structure unextractable by low salt treatment (3, 6, 9). Incubation of muscle fiber bundles with an excess of monovalent anti-M-creatine kinase Fab leads instead to the removal of the electron opaque material from the M-band and to the concomitant release of M-line-bound creatine kinase (1, 8). The binding of creatine kinase to the myofibrillar M-band is isoenzyme specific, i.e. only MM-creatine kinase, and not BB-creatine kinase or the heterodimer MB-creatine kinase, are located at this sarcemere region (6, 7, 10). These observations, when taken together with the molecular dimensions of creatine kinase and the amount of creatine kinase extractable from the M-band, led to the conclusion that creatine kinase is the principal component of m-bridges and thus is also a structural protein (6, 8). The presence of MM-creatine kinase at a specific location within the contractile apparatus suggests a possible catalytic function for the bound enzyme in addition to its structural role (4, 11–15).

The present work provides direct experimental evidence for an enzymatic function and for the physiological significance of the M-line-bound creatine kinase. We demonstrate that the bound creatine kinase acts as a potent intramyofibrillar ATP-regenerating system. These findings support a functional coupling, within the contractile apparatus, of the M-line-bound creatine kinase with the myofibrillar actin-activated Mg2+-ATPase (15).

MATERIALS AND METHODS

Reagents

AMP, ADP, ATP, and Ap-5-A' were obtained from Boehringer-Mannheim, rabbit creatine kinase, carboxatractylsido, and atracyloside from Sigma. Phosphorylcreatine was purchased from Calbi-chem-Behring. Solutions of ATP and phosphorylcreatine freshly prepared and stored frozen at neutral pH for short periods of time proved most satisfactory for the critical pH-stat calibration work. DNFB was obtained from BDH Chemicals Ltd. and PMSF from Serva. EGTA, EDTA, β-mercaptoethanol, and IAA were obtained from Fluka.

Determination of Creatine Kinase Activity

A modification of the indirect enzyme-linked spectrophotometer assay for creatine kinase (16) as described (6) or the direct determination of phosphorylcreatine transphosphorylation in the pH-stat was used to measure the reverse creatine kinase reaction (ADP + phosphorylcreatine → ATP + creatine). The first assay was carried out in 1-ml cuvettes in a Gilford 2400 spectrophotometer at 25 °C. The assay mixture contained 40 mM glucose, 30 mM MgCl2, 1.5 mM ADP, 2 mM AMP, 1 mM NADP, and 0.3 mM hexokinase, and 0.2 u.e. of creatine kinase, respectively.

1The abbreviations used are: Ap-5-A, (p',p'-di(adenosine-5'-)pentaphosphate; IAA, iodoacetate acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; DNFB, 2,4-dinitro-1-fluorobenzene; PMSF, phenylmethylsulfonyl fluoride; GaRF, fluorescein isothiocyanate-conjugated goat anti-rabbit IgE.
of glucose 6-phosphate dehydrogenase in 0.1 M triethanolamine buffer, pH 7.2. The reaction was started by addition of phosphorylcreatine to a final concentration of 6.5 mM. The samples were diluted with buffer containing 0.2 mg/ml of bovine serum albumin in order to keep the ΔA at 340 nm below 0.04/min.

Since the direct pH-stat assay a sample containing creatine kinase activity was added to 5 or 10 ml of assay mixture containing 75 mM KCl, 10 mM MgCl₂, 0.1 mM EGTA, and 4 mM ADP, pH 7.0, at 25 °C. The reaction was started by the addition of 10 mM phosphorylcreatine, 20 mM HCl was used as a titrant. Calibration of the molar ratio of H⁺ utilized per phosphorylcreatine transphosphorylated at different pH values was achieved with purified chicken MM-creatine kinase by limiting either phosphorylcreatine or ADP in the assay mixture and letting the reaction go to completion. For this purpose freshly prepared phosphorylcreatine or spectrophotometrically determined ADP stock solutions were used (E₂₆₀ of ADP at 260 nm = 15,400 cm⁻¹). To prevent inactivation of purified enzyme by dilution, 1 mg/ml of bovine serum albumin was added to the reaction mixture and 0.1–1 mM β-mercaptoethanol was present in the samples.

**Determination of Adenylate Kinase**

Myokinase (ATP:AMP phosphotransferase, EC 2.7.4.3) was measured spectrophotometrically by the coupled assay used for creatine kinase but omitting phosphorylcreatine and AMP. The reaction was started by addition of ADP. In all these assays 1 enzyme unit is defined as the amount of enzyme converting 1 μmol of substrate/min at 25 °C.

**Determination of the Actin-activated Mg²⁺-ATPase**

Direct pH Stat Assay with ATP as a Substrate—The actin-activated Mg²⁺-ATPase activity was determined directly by the pH-stat method described (17), using a Radiometer RTS 822 autoburette. 5 or 10 ml of assay mixture (75 mM KCl, 10 mM MgCl₂, 0.1 mM EGTA, pH 7.0) were introduced into the double glass reaction vessel thermostated at 25 °C. To prevent accumulation and attachment of myofibrils to the glass wall, the assay medium was stirred by a magnetic stirrer and the whole unit mounted on a horizontally rotating shaker (Paramix II, Julabo). The reaction was started after 1–2 mg of myofibrils had been introduced to the assay mixture by the addition of ATP to 4 mM and the activity in the absence of Ca²⁺ was recorded for 10–15 min. Subsequently, CaCl₂ was added to give a final concentration of 0.2 mM and the activity recorded again for 10–15 min. The titrant was 20 mM NaOH. The molar ratio of OH⁻ consumed per ATP hydrolyzed at different pH values, pH 6.2–7.9, was determined by limiting the ATP concentration; aliquots of a freshly prepared, spectrophotometrically determined (E₂₆₀ of ATP at 260 nm = 15,400 cm⁻¹) ATP stock solution were added to either IAA-treated myofibrils or to reconstituted actomyosin and the reaction was allowed to go to completion.

**Combined Creatine Kinase/ATPase pH-Stat Assay with Phosphorylcreatine Plus ATP as Substrates**—The actin-activated Mg²⁺-ATPase of washed myofibrils was also measured by a combined pH-stat assay system similar to that described in Ref. 18. Their assay was designed to maintain ATP concentrations constant during ATPase measurements by the addition of phosphorylcreatine and creatine kinase as a backup system. Overall, phosphorylcreatine is transphosphorylated and protons are consumed (18). In our case, however, the reaction was first measured without adding any exogenous creatine kinase. Typically, the reaction was started by the addition of 1–2 mg of myofibrils to the assay mixture containing 75 mM KCl, 10 mM MgCl₂, 0.1 mM EGTA, 4 mM ATP, and 10 mM phosphorylcreatine at pH 7.0 and 25 °C. The titrant used was 20 mM HCl. The first measurement was made with 20 mM EGTA for 10 min and subsequently again after addition of CaCl₂ to 0.2 mM. After recording for 10–15 min, the combined ATPase/creatine kinase overall reaction driven only by endogenous M-line-bound creatine kinase, an excess of exogenous creatine kinase was added and the new steady state rate measured.

**Preparation of Myofibrils**

Pectoralis muscle from chicken, cut into small pieces immediately after killing of the animal, was transferred into Solution A (0.1 M KCl, 1 mM EGTA, 5 mM EDTA, 0.1 mM β-mercaptoethanol, 0.1 mM PMSE, and 3 mM NaN₃ at pH 7.0) containing 50% glycerol. After penetration of the glycerol (about 1 h) the pieces were transferred into a Petri dish filled with Solution A at 4 °C. Connective tissue was removed and the pieces teased into 1-2-mm thick and 5-30-mm long fiber bundles which subsequently were homogenized in the 50-m1 attachment of the Sorvall Omni-Mixer for 3 × 7 s at full speed. After centrifugation for 7 min at 800 × g, the myofibrils were resuspended in 20 volumes of Solution A, homogenized once for 7 s, filtered through nylon gauze (Seyvlon 180 N) to remove connective tissue and non-homogenized material, and allowed to stand on ice for 20 min prior to centrifugation at 1500 × g for 7 min. This washing procedure, best suited for chicken pectoralis muscle to remove soluble creatine kinase as well as mitochondria and membrane fragments (see “Results”), was repeated eight times with Solution A as a buffer and then twice with a buffer compatible with the pH-stat assay (75 mM KCl, 2.5 mM MgCl₂, 0.1 mM EGTA, 3 mM NaN₃, 5 mM imidazole at pH 7.0) (17).

**Extraction of M-line-bound Creatine Kinase**

M-line-bound creatine kinase was extracted from washed myofibrils either by treatment with 20 % (w/v) of 5 mM Tris/HCl, pH 7.8, for 15–45 min at 4 °C (3, 9) or by incubation with an excess of monovalent anti-M-creatine kinase Fab antibodies (1, 8) both of which remove M-line-bound creatine kinase and concomitantly extract the electron density of the M-line structure (1, 8).

**Inhibition of M-line-bound Creatine Kinase Activity**

Specific anti-M-creatine kinase IgG and Fab, characterized as described earlier were used (1, 6–8) after affinity purification and further characterization by immunoblotting (10).

**Other Procedures**

Adenylate kinase activity was inhibited by preincubation of myofibrils with 1 mM ATP–5 mM for 30 min before the pH-stat assay (21). Mitochondrial ATP/ADP translocase was inhibited by preincubation of myofibrils at pH 4 °C with 200 μM atractyloside or 50 μM carboxyatractysloside for 30 min before the pH-stat assay (22). Mitochondrial respiration was blocked by NaN₃ or KCN that were added to the ATPase/creatine kinase overall reaction assay mixture to a final concentration of 5 mM and 2.5 mM, respectively. Indirect immunofluorescence of myofibrils using anti-M-creatine kinase IgG and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Cappel) were performed as described earlier (1, 6–8, 10). Protein was determined by the method of Lowry et al. (23).

**RESULTS**

**Calibration and pH Optima of Creatine Kinase, Actin-activated Mg²⁺-ATPase, and Combined Creatine Kinase/ATPase Reactions**—For the calibration of the combined creatine kinase/ATPase reaction (e.g., consumption of protons/mol of phosphorylcreatine and ATP hydrolyzed as a function of pH) the creatine kinase and actin-activated Mg²⁺-ATPase reactions were first calibrated separately. Proton consumption per transphosphorylated phosphorylcreatine (creatine kinase reverse reaction, see “Materials and Methods”) was virtually independent of pH between pH 6 and 8 (Fig. 1). The presence of ATP at 4 mM ATP (the concentration used in the combined creatine kinase/ATPase assay) did not affect the calibration at pH 7.0. All subsequent measurements of the combined creatine kinase/ATPase reaction were carried out at pH 7.0, i.e., under conditions in which 1 mol of HCl was consumed per mol of transphosphorylated phosphorylcreatine. In contrast to the creatine kinase reaction, NaOH consumption per hydrolyzed ATP in the actin-activated Mg²⁺-ATPase reaction showed a marked pH-dependence between pH 6.0 and 8.0 (Fig. 1). At pH 7.0, the consumption of NaOH (i.e., the liberation of protons) was 0.67 mol/mol of ATP hydrolyzed. The addition of 10 mM phosphorylcreatine as was present in
phosphorylcreatine with a concomitant consumption of 0.33 mol of phosphorylcreatine, respectively. The components initially added to the assay are framed. Since at pH 7.0, the molar ratio derived from independent calibration of the two reactions is consistent with the value determined directly by limiting phosphorylcreatine in the combined creatine kinase/ATPase reaction (not shown). From Fig. 1 it is obvious that it was difficult to measure accurately by the steady state rates of the combined creatine kinase/ATPase reaction at pH values approaching pH 7.5 where the pH optimum for the activated Mg$^{2+}$-ATPase would be (Fig. 2) because the net consumption of protons ($bH^+ - aH^+$) approached zero. Therefore, it was decided to measure the combined creatine kinase/ATPase reaction routinely at pH 7.0 where the myofibrillar ATPase and creatine kinase both were about 80% of maximum (Fig. 2). Thus, neither activity was favored unduly. This pH value also corresponds well to that measured in living muscle tissue by NMR (24-26).

What was measured by the coupled pH-stat assay (combined creatine kinase/ATPase reaction) was indeed the actin-activated Mg$^{2+}$-ATPase activity for the following reasons: 1) the assay was linear, not only with the amount of myofibrils added, but also, if exogenous creatine kinase was present in excess, with the amount of actomyosin that was reconstituted from purified myosin and actin at a ratio of 2:1 (w/w) (not shown). 2) The pH-dependence of the myofibrillar ATPase (or of reconstituted actomyosin) measured directly by monitoring ATP hydrolysis overlapped with the pH profiles for the indirect, combined creatine kinase/ATPase assay, in which phosphorylcreatine transphosphorylation was measured (Fig. 2). 3) Reactions measured in both ways showed Ca$^{2+}$-dependence and concomitant calcium sensitivity of ≥ 94% (Fig. 4a, Table 1). 4) Calcium-dependent contraction of myofibrils was observed by light microscopy during the pH-stat assay.

Washing of Myofibrils and Amount of M-line-bound Creatine Kinase—Chicken pectoralis muscle contained 2200 u.e. of creatine kinase/g of wet muscle as measured by the direct pH-stat assay. Assuming a maximal specific activity of 400 u.e./mg of purified chicken MM-creatine kinase as measured by the same assay, the total amount of creatine kinase represents approximately 5 mg/g, wet weight. Repeated washing of the combined reaction had a negligible effect on this calibration at pH 7.0 (not shown).

The combined creatine kinase/actin-activated Mg$^{2+}$-ATPase reaction of myofibrils measured in the presence of ATP, phosphorylcreatine, and Ca$^{2+}$ can therefore be described as follows:

\[
\text{Myofibrils} + Mg^{2+}\text{-ATPase} \xrightarrow{\text{Actin} + \text{Calcium}} ADP + P_i + aH^+ \\
\text{ADP} + \text{phosphorylcreatine} + bH^+ \xrightarrow{\text{Creatine Kinase}} ATP + \text{Creatine}
\]

If creatine kinase (endonogenous M-line-bound or exogenously added) is present in excess, the ADP produced by the actin-activated Mg$^{2+}$-ATPase is rephosphorylated by creatine kinase and the following net reaction can be written: phosphorylcreatine + (bH$^+ - aH^+$) = creatine + P$_i$. (H$^+$ are protons); $a$ and $b$ are molar fractions of protons liberated per hydrolyzed ATP and protons consumed per transphosphorylated phosphorylcreatine, respectively. The components initially added to the assay are framed. Since at pH 7.0, $a$ and $b$ were calibrated to be 0.67 and 1.0, respectively (Fig. 1), the combined net reaction consisted of a transphosphorylation of phosphorylcreatine with a concomitant consumption of 0.33 protons ($bH^+ - aH^+$) per mol of phosphorylcreatine transphosphorylated and a net production of P$_i$, and creatine. The above molar ratio derived from independent calibration of the two reactions is consistent with the value determined directly by limiting phosphorylcreatine in the combined creatine kinase/ATPase reaction (not shown). From Fig. 1 it is obvious that it was difficult to measure accurately by the steady state rates of the combined creatine kinase/ATPase reaction at pH values approaching pH 7.5 where the pH optimum for the activated Mg$^{2+}$-ATPase would be (Fig. 2) because the net consumption of protons ($bH^+ - aH^+$) approached zero. Therefore, it was decided to measure the combined creatine kinase/ATPase reaction routinely at pH 7.0 where the myofibrillar ATPase and creatine kinase both were about 80% of maximum (Fig. 2). Thus, neither activity was favored unduly. This pH value also corresponds well to that measured in living muscle tissue by NMR (24-26).

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of chicken pectoralis myofibrils by differential centrifugation with relaxing buffer of physiological ionic strength led to a homogeneous preparation of myofibrils that was essentially free of mitochondria as judged by phase contrast microscopy and by electrophoretic analysis for the mitochondrial creatine kinase isoenzyme (not shown). After five wash cycles in relaxing buffer, the amount of myofibrillar bound creatine kinase could be seen to level off at about 0.8–0.9 U/mg of myofibrils (Fig. 3). Concomitantly the soluble sarcoplasmic creatine kinase was removed and the amount of creatine kinase released into the supernatants decreased with each wash cycle and was negligible after the fifth wash. The creatine kinase content of untreated myofibrils was taken as 100%. Means were averaged from four experiments. Standard deviations were less than 10%. CP, phosphorylcreatine.

| Treatment | Actin-activated Mg$^{2+}$-ATPase activity measured directly (F) | Calcium sensitivity (G) | Actin-activated Mg$^{2+}$-ATPase activity measured by the combined creatine kinase/ATPase pH-stat assay in the presence of Ca$^{2+}$ (H) | Amount of M-line-bound active creatine kinase (I) |
|-----------|-------------------------------------------------|-----------------|-------------------------------------------------|-----------------|
| Untreated myofibrils (A) | 0.022 ± 0.002 | 0.37 ± 0.02 | 0.37 ± 0.02 | 0.8 ± 0.02 | 100% |
| Myofibrils after 10 mM IAA (B) | 0.099 ± 0.006 | 0.33 ± 0.02 | Not measurable | 0.33 ± 0.02 | 0.01 ± 0.001 | 0.13 |
| Myofibrils after 50 μM DNFB (C) | — | — | — | — | — |
| Myofibrils after 15 min of 5 mM Tris, pH 7.8 (D) | 0.036 ± 0.003 | 0.33 ± 0.02 | Not measurable | 0.33 ± 0.02 | 0.06 ± 0.003 | 7.5 |
| Myofibrils after 45 min of 5 mM Tris, pH 7.8 (E) | 0.040 ± 0.004 | 0.29 ± 0.02 | Not measurable | 0.29 ± 0.02 | 0.03 ± 0.003 | 3.8 |

* — Not determined.

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**TABLE I**

**ATP regeneration potential of M-line-bound MM-creatine kinase in myofibrils. Effect of inactivation or removal of M-line-bound creatine kinase on the ATP regeneration potential of myofibrils**

Myofibrils from chicken pectoralis major after extensive washing in buffer at physiological ionic strength, freed by differential centrifugation of soluble creatine kinase, mitochondria, and membrane debris (A); myofibrils after treatment with 10 mM IAA at 4 °C, pH 7.0, for 12 h (B); myofibrils after treatment with 50 μM DNFB at 4 °C, pH 7.0, for 3 h (C); myofibrils after M-line extraction by incubation with low ionic strength buffer (5 mM Tris, pH 7.8) for 15 min (D) and 45 min (E). Myofibrillar actin-activated Mg$^{2+}$-ATPase activity obtained by direct pH-stat assay of ATP hydrolyzed in the absence and presence of Ca$^{2+}$ is expressed in micromoles of ATP hydrolyzed per min and milligrams of myofibrils (F) (conditions described in text); calcium sensitivity of myofibrils

\[
\left(1 - \frac{\text{ATPase with EGTA}}{\text{ATPase with Ca}^{2+}}\right) \times 100
\]

(Ref. 17) is expressed in per cent (G). Actin-activated Mg$^{2+}$-ATPase activity of myofibrils measured by combined creatine kinase/ATPase pH-stat assay in the presence of Mg$^{2+}$-ATP, phosphorylcreatine and Ca$^{2+}$ before and after addition of exogenous creatine kinase is expressed in micromoles of phosphorylcreatine transphosphorylated per min and milligrams of myofibrils (H); creatine kinase activity of myofibrils measured by direct pH-stat assay expressed in micromoles of phosphorylcreatine transphosphorylated per min and milligrams of myofibrils (I); the creatine kinase content of untreated myofibrils was taken as 100%. Means were averaged from four experiments. Standard deviations were less than 10%. CP, phosphorylcreatine.

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**FIG. 3.** Amount of creatine kinase associated with myofibrils. Creatine kinase activity expressed in micromoles of phosphorylcreatine transphosphorylated per min and milligrams of myofibrils from pectoralis major after 1–10 wash cycles. Washes 1–7, with relaxing buffer (0.1 M KCl, 1 mM EGTA, 5 mM EDTA, 0.1 mM mercaptoethanol, 0.1 mM PMSF, and 3 mM NaN$_3$ at pH 7.0) and washes 8–10 with a buffer that is compatible with the pH-stat assay conditions (75 mM KCl, 2.5 mM MgCl$_2$, 0.1 mM EGTA, 3 mM NaN$_3$, and 5 mM imidazole, pH 7.0). Note that about 0.8 U/mg of creatine kinase activity remained bound per mg of myofibrils.
FIG. 4. ATP regeneration potential of M-line-bound creatine kinase before and after treatment with DNFB. a, ATP regeneration potential of creatine kinase bound at the M-band of washed chicken pectoralis myofibrils. Copy of original pH-stat tracing showing the actin-activated $Mg^{2+}$-ATPase activity measured by the combined creatine kinase/ATPase assay with phosphorylcreatine and ATP as substrates. Protons are consumed as phosphorylcreatine is transphosphorylated. The myofibrillar ATPase is supported first by endogenous, M-line-bound creatine kinase only (C) and then by endogenous plus excess of exogenous, added creatine kinase (F). Blank reaction of assay mixture (75 mM KCl, 10 mM MgCl$_2$, 0.1 mM EGTA, 4 mM ATP, and 10 mM phosphorylcreatine at pH 7.0 (A). Addition of 2 mg of washed myofibrils giving rise to some myofibrillar ATPase activity in the absence of Ca$^{2+}$ (B). Steady state rate after the addition of 0.2 mM Ca$^{2+}$ of the actin-activated $Mg^{2+}$-ATPase activity supported only by the endogenous, M-line-bound creatine kinase (C). Addition of an excess (20 u.e.) of purified MM-creatine kinase (d). Fast recharging reaction (E) by the excess of exogenous creatine kinase of ADP that was present at a low steady state level during the previous reaction phase (C) leading to a new, lower steady state level of free ADP during the next phase. New steady state rate of the actin-activated $Mg^{2+}$-ATPase supported by the endogenous plus the excess of exogenous creatine kinase (F). Note the identical slopes in C and F. b, loss of ATP regeneration potential of myofibrils after treatment with DNFB. Copy of original pH-stat tracing showing the actin-activated $Mg^{2+}$-ATPase activity measured by the combined creatine kinase/ATPase assay. The myofibrillar ATPase is supported first by endogenous, M-line-bound creatine kinase only (C) and then by endogenous plus excess of exogenous, added creatine kinase (F). Blank reaction of assay mixture (A). Addition of 2 mg of washed myofibrils that had been treated with 50 $\mu$M of DNFB to specifically inactivate M-line-bound creatine kinase (B). Addition of 0.2 mM Ca$^{2+}$ (at the beginning of phase C) and subsequent addition of an excess (20 u.e.) of purified MM-creatine kinase (d). Recharging of accumulated ADP (E). New steady state rate of the actin-activated $Mg^{2+}$-ATPase activity driven by the excess of exogenous creatine kinase (F). Note the complete loss of the ATP regeneration potential after inactivation by DNFB of the M-line-bound creatine kinase (C). During phase C (no exogenous creatine kinase added) hydrolysis of ATP by the actin-activated $Mg^{2+}$-ATPase which was not affected by DNFB (see Table I) led to a continuous drop of the pH value which was at pH 6.8 at the end of phase C. Since HCl was used as a titrant, this "negative" activity (production of protons by the myofibrillar ATPase) could not be recorded. Assay conditions are as described in a and under "Materials and Methods."
though the steady state concentration of ADP present during phase C is higher than calculations from equilibrium constants of the creatine kinase reaction in the presence of a large excess of creatine kinase would indicate (27), the data presented here show that, after a certain steady state level of ADP was established, the M-line-bound creatine kinase was sufficient to regenerate the ATP hydrolyzed by the myofibrillar ATPase. Addition of excess soluble creatine kinase lowered the steady state concentration of ADP but did not increase the ATPase activity of the myofibrils. ATP regeneration potential and actin-activated Mg²⁺-ATPase activity, i.e. the steady state rates shown in phase C and F in Fig. 4a, were not significantly altered after preincubation of the myofibrils with 1 mM Ap-5-A and 200 μM atractyloside or 50 μM carboxyatractyloside. Sodium azide at 5 mM or KCN at 2.5 mM, both blockers of mitochondrial respiration, had no significant effect on the rates, nor did an additional washing cycle in which myofibrils were incubated overnight at 4 °C with washing solution containing 1% Triton X-100. After treatment of washed myofibrils with these agents, the rate of phase C was always identical with that of phase F (as in Fig. 4a; not shown here). Thus, myokinase and membrane-bound creatine kinase cannot have contributed significantly to the observed ATP regeneration. In addition, both rates (phase C and F as in Fig. 4a), although changing in absolute terms as a function of pH at which the combined creatine kinase/ATPase assay was performed, were always identical in relative terms, when measured at any set pH between pH 6.6 and 7.4 (not shown). During the combined creatine kinase/ATPase assay the creatine kinase activity and thus the ATP regeneration potential remained associated with the myofibrillar pellet. No significant amount of creatine kinase activity was found in the supernatants.

ATP Regeneration Potential after Inactivation of M-line-bound Creatine Kinase by IAA or DNFB—After treatment of washed myofibrils with reagents that inactivate creatine kinase activity (10 mM IAA or 50 μM DNFB; Ref. 20) the ATP regeneration potential via M-line-bound creatine kinase was completely lost (Fig. 4b). In contrast with washed, but untreated, myofibrils (Fig. 4a, phase C) no transphosphorylation of phosphorylcreatine was observed after addition of Ca²⁺ (Fig. 4b, phase C). Thus, although the endogenous myofibrillar creatine kinase was still bound at the M-band as demonstrated by indirect immunofluorescence (Fig. 5, panel 3, a and b), it was inactivated and therefore not sufficient for ATP regeneration (Table I). Due to continuous hydrolysis of ATP by the myofibrillar ATPase, which was not affected significantly by IAA and DNFB (Table I) a continuous drop in pH was observed during phase C (Fig. 4b). After addition of excess exogenous creatine kinase (Fig. 4b, at point d, where the pH had dropped to 6.7) the ADP which had accumulated during phase C was regenerated very rapidly and after some 2 min a linear steady state reaction was observed reflecting again the actin-activated Mg²⁺-ATPase activity as measured by phosphorylcreatine transphosphorylation in the presence of excess creatine kinase. Thus, blocking of M-line-bound creatine kinase by IAA or DNFB (Table I) abolished the ATP regeneration potential of myofibrils without significantly interfering with the ATPase activity or calcium sensitivity (slopes during phase F in Fig. 4a and b were identical, see also Table I).

ATP Regeneration Potential after Extraction of M-line-bound Creatine Kinase by Low Ionic Strength Buffer—Very similar pH-stat tracings as those shown in Fig. 4b were obtained with myofibrils treated with low ionic strength buffer (5 mM Tris/HCl, pH 7.8) which is known to extract the M-line-bound creatine kinase (1, 3, 6). Removal of bound creatine kinase was monitored by direct measurement of creatine kinase activity (Table I) and immunofluorescence staining (Fig. 5, panel 4, a and b). The amount of creatine kinase still remaining at the M-band after extraction by low ionic strength buffer depended on the duration of the treatment. Approximately 92 and 96% of bound creatine kinase was extracted by treatments of 20 and 40 min, respectively. The
endogenous creatine kinase remaining bound at the M-band was not sufficient to keep up with ATP hydrolysis, even though ATPase and calcium-sensitivity were both lowered slightly by prolonged extraction with low ionic strength buffer (Table I). Thus, specific extraction of M-line-bound creatine kinase, like inhibition of bound creatine kinase by IAA or DNFB, abolished the ATP regeneration potential of myofibrils.

Effect of Anti-M-Creatine Kinase Antibodies on the ATP Regeneration Potential—Excess of monospecific anti-M-creatine kinase IgG had a strong inhibitory effect on the myofibrillar bound creatine kinase. As measured by direct pH-stat assay, creatine kinase activity was lowered to about 20% of the control value obtained with preimmune IgG (Table II). Creatine kinase remained associated with the M-band as shown by indirect immunofluorescence staining (Fig. 5, panel 1, a and b). Inhibition of endogenous M-line-bound creatine kinase by anti-M-creatine kinase IgG, like inactivation by IAA and DNFB or extraction of creatine kinase by low salt, also resulted in a loss of ATP regeneration potential without significantly affecting the ATPase activity (Table II). After addition of excess exogenous creatine kinase, a linear steady state activity similar to that of control IgG-treated myofibrils was measured. That is, pH-stat tracings similar to those in Fig. 4b were obtained with anti-M-creatine kinase IgG-treated myofibrils (not shown). In contrast to the results with intact antibody, an excess of monovalent anti-M-creatine kinase Fab fragments not only abolished most of the creatine kinase activity (Table II), but also extracted specifically the M-line-bound creatine kinase as shown by immunofluorescence and pH-stat measurements (Fig. 5, panel 6, a and b; Table II) (Ref. 1). Treatment of pectoralis myofibrils with excess anti-M-creatine kinase Fab, followed by washing to remove MM-creatine kinase-Fab complexes, also resulted in a loss of endogenous ATP regeneration potential of myofibrils (Table II, pH-stat tracing similar to Fig. 4b, not shown). The creatine kinase still remaining bound to the M-band after such a treatment (approximately 8% of the creatine kinase that was bound originally to the M-band, Table II) was not sufficient to keep up with the rate of ATP hydrolysis that was shown to be unimpaired after addition of excess exogenous creatine kinase (pH-stat tracing similar to Fig. 4b, not shown). Incubation with control IgG or Fab did not interfere with M-line-bound creatine kinase activity and had no effect on the ATP regeneration potential (Table II, pH-stat tracing similar to Fig. 4a, not shown).

Immunofluorescence—The effects of the various treatments on the binding of creatine kinase to the M-band are summarized in Fig. 5. Indirect immunofluorescence staining with anti-M-creatine kinase IgG performed with the very same myofibrils that were used for the pH-stat assays revealed that in washed, untreated myofibrils creatine kinase is bound exclusively at the M-band (Fig. 5, panel 1, a and b) (6, 8) that neither IAA or DNFB, nor anti-creatine kinase IgG, affected creatine kinase binding to the M-band (Fig. 5, panels 1, a and b and 3, a and b). However, incubation with low ionic strength buffer (Fig. 5, panel 4, a and b) or excess monovalent anti-M-creatine kinase Fab (panel 5, a and b) did dissociate most of the creatine kinase from the M-band as judged by the loss of regular, cross striated fluorescence patterns (panels 4, a and b and 6, a and b) (1, 8). Control Fab followed by anti-M-creatine kinase IgG did not affect M-line-bound creatine kinase, and bright fluorescence similar in intensity to that with anti-M-creatine kinase IgG alone was observed (panel 5, a and b).

Comparison of the ATP Regeneration Potentials of Different Muscle Types—Depending on muscle type, washed myofibrils prepared under identical conditions contained variable amount of myofibrillar creatine kinase. Pectoralis major and posterior latissimus dorsi from chicken, both fast twitch muscles, showed a higher actin-activated Mg**-ATPase activity than the slow tonic anterior latissimus dorsi or chicken heart muscle, and they also contained more myofibrillar creatine kinase (Table III). The ATP regeneration potentials of the fast and slow skeletal myofibrils were sufficient to keep up with the ATPases, whereas chicken heart myofibrils, which are known to lack a clear electron dense M-band structure

### Table II

**Effect of anti-M-creatine kinase antibodies on the ATP regeneration potential of M-line-bound creatine kinase**

| Myofibrils treated with | Actin-activated Mg**-ATPase activity measured by the combined creatine kinase/ATPase assay (F) | Amount of M-line-bound active creatine kinase (F) |
|-------------------------|------------------------------------------------------|--------------------------------------------------|
|                         | μmol CP/min mg myofibrils | μmol CP/ min mg myofibrils | μmol CP/min mg myofibrils | μmol ATP/min mg myofibrils |
| Control IgG (A)         | 0.35 (0.35)               | 0.79 (100)                        | 0.35 (100)                        | 0.39 (100)                        |
| Control Fab (B)         | 0.37 (0.37)               | 0.76 (96)                         | 0.37 (96)                         | 0.31 (96)                         |
| Anti-M-CK IgG (C)       | Not measurable            | 0.15 (19)                         | 0.34 (19)                         | 0.31 (19)                         |
| Anti-M-CK Fab (D)       | 0.36 (0.36)               | 0.06 (76)                         | 0.36 (76)                         | 0.08 (76)                         |

Note: *Myofibrils from adult mammalian hearts (swine and bovine) show, like chicken skeletal muscle, a sufficient ATP regeneration potential to keep up with the in vitro myofibrillar ATPase.
and M-line-bound creatine kinase (7) did not have sufficient creatine kinase for intramyofibrillar ATP regeneration. However, myofibrils from adult bovine and swine hearts, both of which contain creatine kinase bound at the M-band and display a clearly defined electron-dense M-band structure, were fully competent to regenerate sufficient ATP to keep the actin-activated Mg$^{2+}$-ATPase of these muscles running at their maximal in vitro speeds.\(^1\)

\section*{DISCUSSION}

The results show that ATP regeneration of washed myofibrils is mediated exclusively by the M-line-bound creatine kinase for specific removal by excess anti-MM-creatine kinase Fab of the M-line-bound myofibrillar enzyme resulted in a complete loss of the ATP regeneration potential. Since washed pectoralis myofibrils contained only negligible activities of soluble enzymes and only very few mitochondria, and neither inhibitors of adenylate kinase nor inhibitors of oxidative phosphorylation or of ATP/ADP translocase activity affected the ATP regeneration potential, a significant contribution to ATP regeneration by soluble creatine kinase, adenylate kinase, oxidative phosphorylation, and mitochondrial creatine kinase can be excluded in these experiments.

**ATP Regeneration Potential of M-line-bound Creatine Kinase in Vivo.** The M-line-bound creatine kinase was found to be sufficient in the presence of excess phosphorylcreatine to support the maximal in vitro ATPase activity over a broad pH range, pH 6.4-7.4. Lowering of intracellular pH as much as 0.5 pH units (25) during muscle contraction in vivo brought about by intramyofibrillar hydrolysis of ATP and glycolytic regeneration of ATP (28) will activate further the reaction of M-line-bound creatine kinase in the direction of ATP regeneration (Fig. 2) and increase the intramyofibrillar ATP regeneration potential in vivo. Since 1 g of muscle contains roughly 125 mg of myofibrils (29) and since 55% of the myofibrillar protein is myosin with \(M_\text{x} = 470,000\) (30), the in vitro ATP regeneration potential of the M-line-bound creatine kinase (0.8 \(\mu\)mol of phosphorylcreatine \(\times\) mg myofibrils\(^{-1}\) \(\times\) s\(^{-1}\)) amounts to 1.8 \(\mu\)mol of ATP generated \(\times\) g of wet muscle\(^{-1}\) \(\times\) s\(^{-1}\) at 25°C and pH 7.0. Depending on the muscle type, the maximal power output of skeletal muscle during in vivo contractions at 20°C was measured by chemical analysis to be 1.4 and 3.7 \(\mu\)mol of phosphorylcreatine transphosphorylated (ATP hydrolyzed) \(\times\) g\(^{-1}\) \(\times\) s\(^{-1}\) for rat soleus and extensor digitorum longus, respectively (31). Values, after correction for temperature, ranging from 2 to 4 \(\mu\)mol \(\times\) g\(^{-1}\) \(\times\) s\(^{-1}\) were obtained by \(^{31}P\) NMR measurements with contracting frog and toad muscles (25, 32-34) and values of maximal power output of 1.5-3 \(\mu\)mol \(\times\) g\(^{-1}\) \(\times\) s\(^{-1}\) were reported for human muscle (35, 36). The M-line-bound creatine kinase alone can regenerate enough ATP in vitro to support a rate of ATP hydrolysis of some 1.8 \(\mu\)mol \(\times\) g\(^{-1}\) \(\times\) s\(^{-1}\) and hence is able to keep up with an ATP turnover rate of the myofibrillar, actin-activated Mg$^{2+}$-ATPase of more than 6 \(\mu\)mol \(\times\) s\(^{-1}\) \(\times\) g\(^{-1}\) under in vitro conditions at 25°C and pH 7.0. This rate would correspond to 50 \(\pm\) 100%, depending on muscle type, of ATP turnover measured in vivo, indicating that M-line-bound creatine kinase can maintain a steady, locally high concentration of ATP also in vivo. Thus, intramyofibrillar regeneration by M-line-bound creatine kinase could account for most of or even the entire regeneration of ATP required for contraction. Although the myofibrillar ATPase activity, as measured by pH-stat via phosphorylcreatine-transphosphorylation, was linear during supercontrac-

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\(^1\) T. Wallimann, unpublished observations.

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