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THE CREATINE-CREATINE PHOSPHATE ENERGY SHUTTLE

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PERSPECTIVES AND SUMMARY

The endergonic functions of the animal organism are fueled ultimately by ATP which is generated primarily through oxidative metabolism in the mitochondrion and through anaerobic glycolysis. A conventional view is that consumption of energy causes the formation of ADP which returns to the mitochondrion, stimulating it (acceptor effect-respiratory control) to consume oxygen and rephosphorylate the ADP to ATP, which diffuses back to the sites of utilization. That this view is insufficient is shown by several observations.

Studies on muscle contraction have shown little relation between myofibrillar activity energized by myosin ATPase and the availability of ATP. There appears to be insufficient ADP formation during muscle activity to cause significant release from respiratory control even though muscle activity does produce a marked parallel rise in oxygen uptake. On the other hand, there is a close parallel between skeletal and heart muscle activity, the concentration of creatine phosphate, and the activity of creatine phosphokinase. The creatine phosphate shuttle was proposed to explain why when a diabetic individual exercised, the blood glucose level and general metabolism were altered exactly as if a dose of insulin had been given. In all regimes for diabetics the amount and timing of exercise is taken into account in determining dosage of insulin.

It was proposed in 1960 (1) that insulin acted to stimulate all endergonic reactions by attaching hexokinase to mitochondria. This could provide for more efficient respiratory control and availability of ATP. Figure 1 shows the proposal as depicted in 1966 (2). Exercise causes a liberation of creatine from contracting muscle fibers. This creatine moves to the mitochondria where nascent ATP would produce creatine phosphate and immediately return ADP, stimulating oxygen uptake, at the same time increasing the flux of creatine phosphate to the muscle fiber. (Figure 2).

In the original proposal there were three parts to the creatine phosphate system of respiratory control and energy delivery.

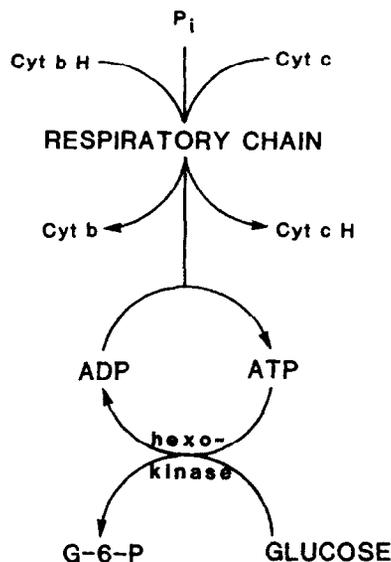


Figure 1 Respiratory control is made more efficient when insulin connects hexokinase to the mitochondrion causing glucose phosphorylation to replace ADP as rapidly as it is formed at certain sites on the mitochondrion.

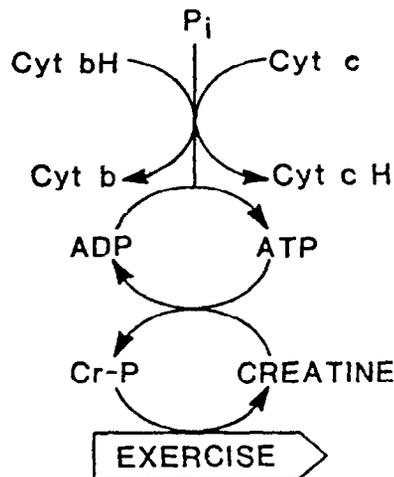


Figure 2 The role of exercise, accelerating oxygen uptake, mimics the effect of insulin in attaching hexokinase to the mitochondrion. This occurs because creatine is liberated by the contracting myofibril, moves to the mitochondrion and there consumes ATP and replaces ADP in situ. The creatine phosphate returns to the myofibril to fuel further contractions.

1. The mitochondrial end which received the creatine "signal," was stimulated by it, and returned the activated (phosphorylated) creatine.
2. The intervening space of diffusion of creatine and creatine phosphate between mitochondrion and every utilization point.
3. The peripheral utilization points where bound CPK would rephosphorylate nascent ADP and allow return of free creatine to the mitochondria.

The concept of the creatine phosphate energy shuttle brings together and sets in context the excellent observations by many workers that were unexplainable by conventional muscle energetics. Its novelty is attested to by the fact that to date no textbook or monograph on muscle contraction refers to the creatine system other than as a reservoir of energy.

CREATINE KINASE

Lohmann (3) recognized the creatine kinase reaction while studying the chemistry of muscle contraction. He found that liberation of creatine during muscle contraction required ADP as a cofactor, and proposed that creatine phosphate reacted with ADP to produce ATP and creatine. Lehmann (4, 5)

showed that the reaction was reversible and subsequently determined the equilibrium values for the reaction. The enzyme was progressively purified (6-8) and Kuby et al crystallized it from rabbit muscle (9).

In 1964 Burger et al (10) described three isozymes of creatine kinase, separable on agar gel electrophoresis. Type I isozyme was found in brain, Type III isozyme was found in skeletal muscle, and Type II isozyme, with intermediate electrophoretic properties, was found in smooth muscle and heart. In the same year Jacobs et al (11) discovered a fourth electrophoretically distinct isozyme of creatine kinase present in mitochondria isolated from brain, heart, and skeletal muscle.

Dance & Watts (12) analyzed the amino acid content of muscle creatine kinase. Half of the expected number of peptides were present after tryptic digestion, suggesting that creatine kinase was a dimer composed of two similar subunits. Dawson et al (13), using chicken brain, muscle, and heart, showed that creatine kinase was a dimer. They found that the three isozymes of creatine kinase described by Burger et al were composed of combinations of two subunits: the B subunit, found in brain, and the M subunit, found in muscle.

The brain isozyme of creatine kinase (Burger's type I) was found to be composed of two B subunits and the muscle enzyme (Burger's type III) of two M subunits. The third isozyme (Burger's type II) was composed of one M and one B subunit.

Eppenberger et al (14) showed that the two subunits were immunologically distinct, that their peptide maps and amino acid composition were distinct, and that the isozymes were kinetically different. They also showed that the isozymes were of similar molecular weight and that the MB form could occur *in vitro* by association of M and B subunits (15).

Creatine kinase is present in the cytosol and mitochondria of heart, skeletal muscle, and brain of vertebrates. Creatine kinase activity is also found in tumors (16), adipose tissue (17), white blood cells (18), and smooth muscle (19). Minimal activity has been found in many other tissues (20). Studies of the function of creatine kinase, the focus of this review, have been done primarily on heart and skeletal muscle.

In general, the mitochondrial isozyme is found in mitochondria isolated from vertebrate heart, skeletal muscle, and brain. The only other isozyme found in brain is the BB isozyme. In normal skeletal muscle the only additional isozyme is the MM form. Heart contains primarily the MM form but in most species, significant amounts of both the MB and BB forms are also present. The chicken heart is an exception; it contains only the mitochondrial and BB forms.

Invertebrate tissues contain an analogous enzyme: argininephosphokinase. Arginine phosphate, also a guanidino phosphate, seems to serve in invertebrates as creatine phosphate does in vertebrates (21). Arginine kinase which

catalyzes the transphosphorylation reaction between arginine phosphate and ATP is also reversible (22).

CREATINE AND CREATINE PHOSPHATE

Creatine is synthesized in two sequential reactions; guanidinoacetic acid is formed from the transguanidination of arginine and glycine, and then *N*-methylated to form creatine. These reactions occur primarily in liver, pancreas, and kidney. The amount of each enzyme present in various tissue differs with the species. Neither enzyme has been found in skeletal muscle, heart, or brain, the tissues which contain the highest concentrations of creatine and creatine phosphate. Both creatine and creatine phosphate react nonenzymatically to form creatinine which is not reconvertible to creatine in significant amounts. These tissues must therefore replenish their creatine pools from the plasma in which it is normally about 0.40 millimolar. The mean muscle concentration of creatine at rest is approximately 5.0 millimolar and the concentration in red blood cells is about 0.5 millimolar.

Thunberg (23) was the first to observe that creatine was involved in muscle metabolism. In 1911 he showed that creatine added to a muscle mince stimulated oxygen consumption. Eggleton & Eggleton (24) found that muscle contained a highly acid-labile organic phosphate. Fiske & Subbarow (25) discovered the compound at about the same time and proved that it was a phosphorylated derivative of creatine. The interest in creatine phosphate, or "phosphagen" focused on its role in energy metabolism.

Meyerhof & Lohmann (26) showed that creatine phosphate had a large free energy of hydrolysis (12 kcal/mol), suggesting it might serve as the source of energy for muscle contraction. Lipmann & Meyerhof (27) established that creatine was liberated during muscle contraction, indicating that the hydrolysis of creatine phosphate was indeed coupled to muscle contraction. Lundsgaard (28) showed that creatine phosphate could be synthesized aerobically.

Almost thirty years after Thunberg's report (23), Belitzer & Tsybakova (29) showed that creatine phosphate synthesis in a muscle mince was coupled to oxygen consumption in the oxidation of 3 and 4 carbon compounds. Thus oxidative phosphorylation was first discovered as a function of creatine.

ATP was discovered in 1929 by Fiske & Subbarow (30). Much evidence accumulated indicating that hydrolysis of ATP provided the ultimate source of energy for muscle contraction. Myosin was found to be an ATPase (31) and it was shown that ATP would cause actomyosin fibrils to contract in solution (32). No one could demonstrate, however, that ATP was hydrolyzed by intact muscle during contraction. This led A. V. Hill (33) to challenge biochemists to prove that ATP truly supplied the energy for muscle contraction. A decade later

Cain & Davies (34) demonstrated a decrease in ATP levels in contracting muscle that had been treated with 2,4-dinitrofluorobenzene to inhibit creatine kinase completely. Although this experiment established the fact that ATP hydrolysis is the immediate source of energy for muscle contraction, it also emphasized the close association of creatine kinase and creatine phosphate with the contractile process, a fact that was not considered in subsequent discussions of the energetics of muscle contraction.

COMPARTMENTATION OF CELLULAR ENERGY METABOLISM

The creatine phosphate energy transfer system is an example of metabolic compartmentation: the localization of enzymes and intermediates resulting in enhanced efficiency. There are several recent symposia devoted to metabolic compartmentation (35, 36). Compartments restrict or facilitate the access of substrates to enzymes. By compartmentation an enzyme's environment can be optimized for pH, concentrations of ions, cofactors, substrates, and location of precursor enzymes. Compartmentation allows local variation in these factors to regulate enzyme activity.

Compartments are created when substrate diffusion is restricted either by binding or membrane barriers. The most obvious examples of compartments are those that are membrane delimited, such as mitochondria, nuclei, and lysosomes. Nevertheless the proximity or binding of sequential enzymes in the same metabolic pathway may also limit diffusion because a nascent intermediate is delivered primarily to the active site of the next enzyme in the pathway, such as in the fatty acid synthetase complex. Binding of intermediates also results in compartmentation, as in the glyceraldehyde phosphate dehydrogenase reaction (37). The acyl intermediate is bound to the enzyme at a tenfold higher concentration than the free compound.

THE CREATINE PHOSPHATE SHUTTLE

The creatine phosphate shuttle explains the role of creatine, creatine phosphate, and creatine kinase in facilitating energy distribution and responding to energy demand. The concept arose from studies of insulin action. Insulin has no effect on the turnover of creatine phosphate or on muscle contraction, but when a diabetic exercises, the blood glucose falls and other chemical changes occur that are the same as those occurring after a dose of insulin. Bessman (38) proposed that insulin acts by causing the binding of hexokinase at strategic mitochondrial sites where it could phosphorylate glucose with nascent ATP and resupply ADP efficiently for respiratory control (Figure 1). From this foundation, and based on subsequent work to be reviewed, Bessman proposed that

creatine from the contracting muscle provided the stimulus for oxygen uptake (Figure 2). This was further refined (39) in 1972. Creatine phosphate is synthesized in mitochondria. The creatine phosphate diffuses to the myofibrils, where the MM isozyme of creatine kinase is bound. As contraction generates ADP, creatine kinase catalyzes the resynthesis of ATP to allow continued contraction. The creatine formed at the myofibril is thus rephosphorylated by mitochondria. This process was named the "Creatine Phosphate Shuttle" in 1978 (39a) and reviewed in detail in 1980 (40).

Gudbjarnason et al (41) proposed that ATP and creatine phosphate are compartmented at sites of utilization, based on the observation that contraction in anoxic heart stops when ATP levels are only minimally decreased, but creatine phosphate is depleted. Apparently unaware of experimental evidence for creatine as the respiratory control mediator in muscle (42) they proposed that adenine nucleotides are compartmented into mitochondrial and myofibrillar pools and creatine phosphate mediates the transfer of energy. Only a portion of total cellular ATP was thought to have access to myofibrils.

The creatine phosphate shuttle is based on the view that adenine nucleotides and creatine kinase are indeed compartmented. Adenine nucleotides are thought to be located primarily in mitochondria and near or bound to peripheral ATPases, e.g. myosin ATPase. Creatine kinase, bound as various isozymes to mitochondria and myofibrils, is thought to have the same peripheral distribution as the ATPases and also to synthesize creatine phosphate in mitochondria from nascent ATP. There are structural and functional data to indicate that this view is correct. There is also evidence that other ATPases in muscle, heart, and brain may derive the ATP they use by the creatine phosphate shuttle using adjacent isozymes of creatine kinase.

There are several reviews of the creatine phosphate shuttle (40, 43) and it has been the subject of symposia in 1979 and 1984 (43, 44).

COMPARTMENTATION OF ENERGY METABOLITES IN HEART AND SKELETAL MUSCLE ADENINE NUCLEOTIDES

Perry (45) long ago proposed that adenine nucleotides might be compartmented. He thought they were localized to myofibrils and that "such a system would have the advantage of maintaining ATP precisely where it is needed for contraction." In subsequent years a great deal of evidence has accumulated that suggests that adenine nucleotides are indeed compartmented. Mommaerts (46), using contracting frog muscle, showed there was no detectable change in ATP or ADP during contraction, and that changes in creatine phosphate alone accounted for the work.

Seraydarian et al (47) have shown that ADP binds to actin. Veech et al (48)

measured concentrations of intermediates of reactions that are presumed to be in equilibrium and in which ADP is a substrate. The free ADP concentration in skeletal muscle, brain, and liver was estimated to be 20-fold lower than the total ADP concentration. They proposed that sequestration in mitochondria may account for low free ADP levels in brain and liver. Provision of ATP formed by ATPases to mitochondria by diffusion must be restricted by the low concentration of ADP, which would limit respiratory control by ATP availability, as pointed out originally by Chance (49).

D. K. Hill (50) used ^3H -labeled adenine to study the distribution of adenine nucleotides in frog muscle. 50–80% of the adenine nucleotides were located at the A-band of the myofibrils. Ottaway & Mowbray (51) suggested that the possibility of artifact limits the conclusions that can be drawn from this study. Lanthanum was used in the fixative. It catalyzes the hydrolysis of ATP and seems to be limited to the extramyofibrillar space.

Gudbjarnason et al (41) studied the effect of ischemia on the concentrations of high energy phosphates in dog heart. Ischemic muscle stopped contracting when 75% of the creatine phosphate had been depleted even though 80% of the ATP remained. A large proportion of the ATP appears not to have access to the myofibrillar ATPase. Dhalla et al (52) and Neeley (53) also found that decreasing cardiac output in ischemic hearts correlated well with creatine phosphate levels and only minimally with ATP levels.

Studies of muscle that have been stimulated to fatigue have shown that a large proportion of the ATP remains and that depletion of creatine phosphate correlates with fatigue (54–56).

Nassar-Gentina et al (57) proposed that a defect in excitation-contraction coupling during ischemia and fatigue would prevent utilization of ATP and might explain conservation of ATP in ischemic and fatigued muscle and heart. After a muscle has been stimulated to fatigue, addition of 5 mM caffeine allowed an additional tetanic contraction to occur during which most of the remaining ATP was hydrolyzed. They suggested that caffeine releases calcium from the sarcoplasmic reticulum, overcoming the defect in excitation contraction coupling. Caffeine is well known to disrupt muscle ultrastructure (59) and probably releases calcium by disrupting the membrane. Since the effect of caffeine is apparently the result of destruction of compartments, the compartmentation of ATP could also have been affected, allowing access of previously compartmented ATP to the myofibrillar ATPase.

Studies of muscle and heart in which creatine kinase has been inhibited with 2,4-dinitrofluorobenzene (FDNB) also suggest that ATP is compartmented. Infante & Davies (60) studied contractions in frog sartorius muscle in which creatine kinase had been completely inhibited by preincubation with FDNB to prevent regeneration of ATP from creatine phosphate. The muscle could be stimulated to contract three or four times with a 50% reduction in ATP. After

ten minutes the muscle would again contract fully and after another ten minutes the muscle would contract weakly with a further decrease in ATP. Although there was no synthesis of ATP during the ten-minute rest periods, some of the normally sequestered ATP became available for contraction, suggesting that ATP compartmentation is not absolute. Over the ten-minute period sufficient ATP must have diffused to the myofibrillar ATPase sites to support additional contraction. Gercken & Schlette (61) measured ATP levels in rat heart perfused with FDNB. At the time of failure 85% of the ATP remained, as did most of the creatine phosphate.

In frog heart, which is permeable to phosphorylated compounds, contractile force decreases with creatine phosphate depletion, even though ATP levels remain at about 75% of the control value (62, 63). Reperfusion with creatine leads to a parallel increase in creatine phosphate and contractile force. ATP increases to 90% of the control value. These results were confirmed by Vassort & Ventura-Clapier (64). These data emphasize the dependence of contractile function on creatine phosphate and the limited access of the total ATP pool to the contractile apparatus, in amphibia as well as mammalia.

McClellan et al (65) used rat heart, made hyperpermeable by EDTA treatment, to study energy transport. An increase in tension of the heart muscle strips resulted from a low energy state. In these nucleotide-depleted tissues, relaxation occurred with creatine phosphate perfusion, confirming that sufficient ADP must have been present at the myofibrils to be rephosphorylated. Tissues perfused with mitochondrial substrates and inorganic phosphate did not relax, indicating that the ADP produced by the myofibrils was not available to the mitochondria for rephosphorylation [cf Chance (49)]. Cells did relax if exogenous ADP was supplied, which presumably did have access to mitochondria.

³¹P NMR has been used to investigate ATP compartmentation on the basis of the fact that a difference in pH of the environment causes differing NMR signals. Nunnally & Hollis (66) found two ATP pools, whereas Busby et al (67) concluded there was only one pool. If ATP compartments were not distinguished by a difference in pH, or if the concentrations of ATP in some compartments were small in relation to other compartments, they would not be detected by the presently insensitive ³¹p NMR methods.

COMPARTMENTATION OF CREATINE AND CREATINE PHOSPHATE

Hill (68) also studied the distribution of tritium-labeled creatine phosphate in frog muscle. He found that almost all of the creatine phosphate was localized in narrow bands along the edges of the I-band of the myofibrils and estimated the level of concentration there to be about 150 mM.

Lee & Visscher (69) studied the distribution of ^{14}C -labeled creatine in perfused rabbit hearts. The specific activity (SA) of creatine phosphate rises more rapidly than that of the "free" creatine pool. On washout the SA of the free creatine drops at three times the rate of the creatine phosphate pool. The data are consistent with the existence of two pools of free creatine. Lee & Visscher proposed that a portion of the free creatine might be bound intracellularly. Savabi & Bessman (70) have shown that creatine is not lost from hearts subjected to anoxia, in which there is almost complete loss of creatine phosphate, for when they are reoxygenated there is regeneration of creatine phosphate even to higher than control levels. We have found that beating atria brought to a standstill in anoxia in which the creatine phosphate is almost completely depleted, do not lose any of the intracellular creatine to the medium over a 2-hour period (Savabi, 70) for on reoxygenation 100% or more of the original creatine phosphate reappeared. This also suggests that when creatine phosphate is hydrolyzed the liberated creatine may be bound. It appears likely that the chemical activity of intracellular creatine may be considerably lower than the measured concentration of nonphosphorylated creatine.

MITOCHONDRIAL CREATINE KINASE AND THE SYNTHESIS OF CREATINE PHOSPHATE

Isolation and Characterization of Mitochondrial Creatine Kinase

Mitochondrial creatine kinase was first described by Jacobs et al (11) in 1964. They discovered an isozyme of creatine kinase electrophoretically distinct from the cytosolic forms of creatine kinase, in mitochondria of heart, brain, and skeletal muscle. Sobel et al (71) confirmed the findings of Jacobs using electrophoretic methods that were more sensitive and resolved the four isozymes of creatine kinase. Vial et al (72) were the first to study the kinetics of the enzyme, using intact mitochondria.

Farrell et al (73) extracted the enzyme from bovine heart mitochondria with inorganic phosphate. Scholte et al (74) localized the mitochondrial enzyme to the outside of the inner mitochondrial membrane. They found that a fraction of sonicated mitochondrial particles lose their creatine kinase activity, presumably those that are inside out. The activity returned with detergent treatment, which they concluded dissolved the membrane and released the creatine kinase.

Jacobus & Lehninger (20) showed that creatine phosphate synthesis from creatine and ATP formed from oxidative phosphorylation was inhibited by atractyloside, an inhibitor of the adenine nucleotide translocase. They showed that creatine kinase did not have access to matrix nucleotides and confirmed the fact (42) that it was an excellent respiratory control signal and that it was located on the outside of the inner membrane of the mitochondrion.

Hall, Addis, and DeLuca (75, 76) have purified mitochondrial creatine kinase from beef heart and characterized the pure enzyme. They have studied its kinetics, determined the effects of ions and pH on the rate, and determined the amino acid composition. Roberts & Grace (77) purified the mitochondrial isozyme from dog heart and showed it to be immunologically pure. Recently Grace et al (78) have purified human mitochondrial creatine kinase to a specific activity greater than 400 IU/mg.

The mitochondrial isozyme, like the cytosolic isozymes, is a dimer of about 80,000 daltons. The mitochondrial isozyme is distinguished on the basis of kinetic constants, and its migration toward the anode on cellulose acetate membrane electrophoresis in Tris barbital at pH 8.8.

FUNCTION OF THE MITOCHONDRIAL ISOZYME

The creatine phosphate shuttle theory proposed that the function of the mitochondrial isozyme of creatine kinase is to synthesize creatine phosphate from creatine and ATP generated *de novo* at the same time returning ADP to the respiratory system thereby stimulating oxidative phosphorylation. Bessman & Fonyo (42) showed, shortly after the discovery of mitochondrial creatine kinase, that creatine stimulated mitochondrial respiration, suggesting that the proposed relation between exercise and mitochondrial energy generation was correct (1). Creatine, acting as an acceptor for the gamma phosphate of ATP, provided ADP for the mitochondrial membrane to stimulate oxidative phosphorylation. Since then, both kinetic and direct labeling studies have elucidated this functional relationship between oxidative phosphorylation and the mitochondrial creatine kinase reaction.

KINETIC SUPPORT FOR THE MITOCHONDRIAL END OF THE CREATINE PHOSPHATE SHUTTLE

Jacobus & Lehninger (20) confirmed the original findings (42) that creatine functioned in respiratory control and showed that rat heart mitochondria contained enough bound creatine kinase activity to permit them to use all of the ATP generated at maximal respiration to synthesize creatine phosphate. Saks et al (79) confirmed their findings. It is clear that heart mitochondria contain enough creatine kinase to produce sufficient creatine phosphate exclusively to deliver the high energy phosphoryl group to myosin ATPase; there is no enzymatic requirement to rely on diffusion of ATP from mitochondrion to myofibril. They showed that the forward mitochondrial reaction (synthesis of creatine phosphate) was kinetically favored (80). They calculated that mitochondrially generated ATP had preferred access to mitochondrial creatine kinase for creatine phosphate synthesis compared to extramitochondrial ATP

(81). Using oligomycin, they studied ATP transport by the adenine nucleotide translocase as measured by oxygen consumption. Creatine phosphate synthesis increased with increasing transport of ATP (oxygen consumption) even if the concentration of ATP in the incubation medium was increased, suggesting that the de novo synthesized ATP had preferred access to creatine kinase. They concluded that these data demonstrated an association between the adenine nucleotide translocase and creatine kinase. They did not rule out the possibility that increasing concentrations of ATP might have produced more ADP which would stimulate oxygen consumption, however.

A different kinetic (82) approach showed that the apparent K_m of mitochondrial creatine kinase for ATP, when generated by mitochondria, was 37 μM . When ATP was provided by extramitochondrial phosphoenolpyruvate (PEP) and pyruvate kinase, the apparent K_m was 200 μM . The apparent K_m was also 200 μM for the solubilized mitochondrial enzyme supplied with ATP by PEP and pyruvate kinase. The apparent K_m for the solubilized mitochondrial was 145 μM . Product inhibition by creatine phosphate was compared in respiring heart mitochondria to liver mitochondria incubated with solubilized mitochondrial creatine kinase. The apparent K_m for ATP was 5–7-fold lower at each creatine phosphate concentration for the heart mitochondria with bound enzyme. The conclusion drawn from these experiments is that the bound creatine kinase of heart mitochondria is exposed to a higher effective concentration of ATP. It is also protected from added creatine phosphate so that there is a lower effective inhibitory concentration of creatine phosphate near the enzyme. These data do not distinguish between substrate compartmentation or close association of nucleotide translocase with creatine kinase.

The effect of the source of ATP on the rate of creatine phosphate synthesis was also studied (83). The rate of mitochondrial creatine phosphate synthesis was higher when ATP was supplied by oxidative phosphorylation than by PEP and pyruvate kinase incubation with mitochondria inhibited by oligomycin, again supporting the concept of the mitochondrial compartment.

Altschuld & Brierly (84) had earlier reported that the rate of creatine phosphate synthesis was unaffected by the source of ATP. They compared respiring mitochondria with mitochondria inhibited with atractyloside and incubated with PEP and pyruvate kinase. The rate of oxygen consumption, and thus ATP synthesis by the mitochondria, was low. Limited ATP synthesis probably decreased the rate of creatine phosphate synthesis in their respiring beef heart mitochondria.

Moreadith & Jacobus (85) took a similar approach in studying the apparent K_m of ADP for respiration. They found that ADP provided by the mitochondrial creatine kinase reaction had an apparent K_m for respiration of 2–4 μM . ADP provided by extramitochondrial glucose, ATP, and hexokinase had an apparent K_m of 19–20 μM . To further test if ADP generated by the mitochondrial

creatine kinase reaction results in a higher concentration of ADP near the translocase, they compared the effect of the source of ADP on a competitive and noncompetitive inhibitor of the adenine nucleotide translocase. They found that atractyloside (a competitive inhibitor) was less effective in inhibiting respiration when the ADP was provided by the creatine kinase reaction, confirming that the effective concentration of ADP near the translocase is higher in this system. To try to identify whether the outer mitochondrial membrane or an association between creatine kinase and the adenine nucleotide translocase was responsible for the compartmentation, they compared ADP generated in the intermembrane space by nucleoside diphosphokinase (NUDIKI) to that generated by creatine kinase. Liver mitochondria contain NUDIKI in the intermembrane space. UDP was added to act as a phosphate acceptor and regenerate ADP to stimulate respiration. The ability of atractyloside to inhibit respiration under these conditions was compared to its inhibition when ADP was generated by the extramitochondrial hexokinase reaction. Atractyloside inhibited respiration equally for ADP generated by NUDIKI or hexokinase. In heart mitochondria, atractyloside was less effective in inhibiting respiration stimulated by ADP provided by the creatine kinase reaction. They concluded that the compartmentation is conferred by an association between the adenine nucleotide translocase and creatine kinase and not the mitochondrial outer membrane, since ADP generated in the inner membrane by NUDIKI was less effective at competing with atractyloside. A close examination of the data reveals that this was not a fair comparison. In the absence of atractyloside the maximum rate for NUDIKI-stimulated respiration was only 67% of the maximal rate when ADP was added directly to stimulate respiration. ADP generation limited respiration in the experiment with NUDIKI, whereas in heart mitochondria there was a minimal difference in the rates of respiration stimulated by creatine and that stimulated by added ADP. Since NUDIKI is unable to generate sufficient ADP to drive mitochondria to their maximal respiratory rate it is not valid to compare its ability to generate locally high concentrations of ADP to that of creatine kinase, which under the same experimental conditions is able to drive mitochondria to their maximal respiratory rate.

Gellerich & Saks (86) also studied ADP provision for mitochondrial respiration. They used PEP and pyruvate kinase to consume extramitochondrial ADP and showed that respiration was not increased when ADP was added directly or through the hexokinase reaction. When provided by ATP and creatine through the creatine kinase reaction, ADP stimulated respiration. This experiment emphasizes the fact of compartmentation of adenine nucleotides in mitochondria but does not define the nature of the compartment as a direct association between the translocase and creatine kinase. Jacobus & Saks (87) found that ADP supplied to bound mitochondrial creatine kinase by oxidative phosphorylation resulted in a 2–3-fold decrease in the K_a of ATP for the ternary complex

E·MgATP, but a tenfold decrease in the K_a of ATP for the ternary complex E·MgATP creatine. Provision of ATP through oxidative phosphorylation stabilizes the ternary complex, compared to ATP from PEP and pyruvate kinase. If compartmentation were due to a localized increase in the concentration of ATP, the decrease in the K_a of ATP for the binary and ternary complexes should be equal. This suggests that the bound enzyme kinetics are not rapid-equilibrium random binding, as is found with the solubilized enzyme. Applying the fact that phosphate ion solubilizes mitochondrial creatine kinase (87a) Hall & DeLuca (87b) showed that increases in inorganic phosphate in the physiological range diminished the preferential use of mitochondrial ATP in synthesis of creatine phosphate.

DIRECT ANALYSIS OF MITOCHONDRIAL COMPARTMENT LABELING

Labeling studies have used either $^{32}\text{P}_i$ inorganic phosphate or gamma $^{32}\text{P}_i$ -labeled ATP directly to assess the contribution of ATP synthesized de novo to mitochondrial creatine phosphate synthesis. These studies have been done primarily in our laboratory.

Yang et al (88) used gamma $^{32}\text{P}_i$ -labeled ATP incubated with respiring rabbit heart mitochondria and measured the amount and specific activities of ATP and creatine phosphate at 5 and 10 sec. The specific activity of creatine phosphate was less than half that of ATP indicating that a large amount of the creatine phosphate had been synthesized from mitochondrial ATP. When mitochondrial ATP synthesis was inhibited by the uncoupler carbonyl cyanide chlorophenylhydrazine (CCCH) or atractyloside the specific activity of the creatine phosphate was equal to that of the added ATP, indicating no mitochondrial contribution.

Erickson-Viitanen et al (89) studied in more detail the contribution of mitochondrial ATP to the synthesis of creatine phosphate in isolated mitochondria. Analysis of these experiments rests on consideration of two distinct ATP pools: that present in the medium and that synthesized by mitochondria. Since there was insignificant breakdown of creatine phosphate during the short time course of these experiments and the rate of creatine phosphate synthesis is linear, its specific activity was the algebraic sum of the gamma phosphate contributions from the medium ATP and mitochondrially synthesized ATP. Because of the rapid rate of phosphate transport into mitochondria (90) and its incorporation into newly synthesized ATP, the specific activity of the newly synthesized ATP is closely approximated by the specific activity of the inorganic phosphate, in experiments in which P_i was used as the label. The mitochondrial contribution to creatine phosphate synthesis is calculated using the following equation:

$$\text{fractional mitochondrial contribution} = \frac{(SA_{CP}) - (\overline{SA}_{ATP})}{(SA_{P_i}) - (SA_{ATP})} \quad 1.$$

SA_{CP} is the specific activity of creatine phosphate at the end of the incubation interval, SA_{P_i} is the specific activity of inorganic phosphate (which does not change over the short incubation periods used), and \overline{SA}_{ATP} is the mean specific activity of ATP during the incubation interval.

These experiments revealed that demonstration of a significant mitochondrial component of creatine phosphate synthesis depended on the concentration of ATP added to the medium. At ATP concentrations below 0.2 mM a significant mitochondrial contribution can be seen. Experiments done with gamma-labeled ATP, rather than labeled P_i , give the same results. The ATP concentration at which compartmentation is evident is low; several factors may account for this. The isolation procedures may damage the mitochondria making the outer membrane more permeable to ATP or, *in vivo*, since most of the ATP is nondiffusible, the activity of ATP in the vicinity of the mitochondria may be less than 0.2 mM. [See ref. (39a) for more data on the inaccessibility of the large pool of ATP to the mitochondrial pool.]

The specific activity of creatine phosphate was higher than the mean specific activity of ATP between 5 and 10 seconds indicating preferred access of mitochondrial ATP to creatine kinase. The specific activity of glucose-6-phosphate synthesized by added hexokinase is equal to the mean specific activity of ATP. There is no preferred access of newly synthesized ATP to the extramitochondrial hexokinase reaction. These results are both qualitatively and quantitatively consistent with the kinetic calculations of the previous section.

Erickson-Viitanen et al (91) used gentle digitonin treatment to remove the outer mitochondrial membrane to determine whether the outer membrane was responsible for compartmentation. The mitochondria retained all but about 10% of their creatine kinase activity and respired normally suggesting no major change in the translocase. It was not possible, however, to demonstrate preferred access of newly synthesized ATP to creatine kinase in mitochondria without an outer membrane. Compartmentation of the mitochondrial creatine kinase reaction is apparently due to the outer mitochondrial membrane, which presumably restricts diffusion, resulting in a high concentration of ATP in the intermembrane space. Although it is true that the outer mitochondrial membrane is permeable to adenine nucleotides this does not mean that it does not limit loss of nucleotide at all. The limiting effects of this membrane are worthy of much further study to give insight into the dimensions of this form of compartmentation. These experiments do not support the hypothesis that there is close association between the translocase and creatine kinase. If there were such an association, a mitochondrial contribution to creatine phosphate syn-

thesis should still have been detected in the mitochondria stripped of their outer membrane.

Recent experiments by Barbour et al (91a) using ^3H -labeled nucleotides confirm the role of creatine in respiratory control (42) and agree very well with the report of Erickson-Viitanen et al (91).

When Altschuld & Brierly (84) studied the synthesis of creatine phosphate by beef heart mitochondria using labeled inorganic phosphate they found no evidence for compartmentation. However, they used 3 mM ATP in the incubation medium, which, as shown above, masks the mitochondrial contribution. They also conducted their experiments for a minimum of 1 min. In our hands creatine phosphate synthesis is not linear over this long a time period.

Lipskaya et al (92) have also used labeled inorganic phosphate to study mitochondrial creatine kinase. In their first experiment, the specific activity of creatine phosphate lagged behind that of ATP. This is as expected, since the mean ATP specific activity from which accumulating creatine phosphate is formed must be compared to the creatine phosphate activity found to find the apparent mitochondrial contribution to creatine phosphate synthesis. In a second experiment they found that by 2 min the specific activity of creatine phosphate exceeded that of ATP. We have found that by 5 min the specific activity of creatine phosphate approaches that of ATP, but never exceeds it. If creatine phosphate accumulates as ATP turns over we do not see how such a result could be achieved. If both creatine phosphate and ATP turned over equally then by 5 min both should be equally labeled. The only way that creatine phosphate activity could exceed ATP activity is if ATP did not turn over as fast as CP.

Both kinetic and direct labeling experiments demonstrate that mitochondrial creatine kinase uses newly synthesized mitochondrial ATP to synthesize creatine phosphate. This compartmentation is due to the outer mitochondrial membrane.

CREATINE PHOSPHATE SYNTHESIS AND GLYCOLYSIS

Early work on creatine phosphate revealed that it is synthesized under anaerobic conditions, presumably through glycolysis (93). When creatine was added to a cytosolic fraction from rat hearts that contained the glycolytic enzymes, glycolytic cofactors and substrates, the production of lactate was accompanied by the synthesis of creatine phosphate. When fructose-1,6-biphosphate was used as the substrate, the ratio of creatine phosphate to lactate formed was about 2, the expected ratio if all of the ATP formed were converted to creatine phosphate (94). Higher concentrations of creatine phosphate inhibited glycolysis, presumably because ADP was converted to ATP as the reactions went to equilibrium. These results can be accounted for by the contamination of the

fraction with creatine kinase. No one has yet shown an association of creatine kinase with the glycolytic enzymes.

THE USE OF CREATINE PHOSPHATE TO REGENERATE ATP

The creatine phosphate shuttle proposes that the cytosolic isozymes of creatine kinase are located very near the ATPases and catalyze the resynthesis of ATP in situ for use by the ATPases. Three major cell functions that require ATP have been shown to be terminals for the creatine phosphate shuttle: contraction, macromolecular synthesis, and maintenance of ion gradients.

CONTRACTION AND CREATINE PHOSPHATE

Binding of Creatine Kinase to Myofibrils

Botts & Stone (95) discovered that myosin ATPase activity was lower in the presence of creatine kinase and proposed that there was an interaction between the two enzymes. Yagi & Mase (96) described noncompetitive inhibition of myosin ATPase by creatine kinase, suggesting interaction of the two enzymes. Ottaway (97) isolated creatine kinase from ox heart myofibrils, indicating that in vivo creatine kinase was bound to myofibrils. Scholte et al (74) fractionated rat heart and skeletal muscle and also found creatine kinase activity in myofibrils.

The site of the binding of CK remains incompletely explained. Turner et al (98) showed that the M-line protein of myofibrils was in part composed of the MM isozyme of creatine kinase. Immunologic studies of chicken heart and skeletal muscle showed that creatine kinase was localized to the M-line in these tissues (99, 100). Herasymowych et al (101) eluted the MM isozyme of creatine kinase from bovine heart myofibrils at low ionic strength and showed rebinding of the M-line. Mani & Kay (102) isolated a 165,000-dalton M-line protein that is a competitive inhibitor of creatine kinase and binds creatine kinase in a one to one ratio.

Houk & Putnam (103) used fluorescence polarization measurement of tagged creatine kinase and found interaction with the rod portion of myosin. Botts et al (104), using more sensitive techniques, found interaction of creatine kinase with intact myosin, heavy meromyosin, and subfragment one. These results were confirmed by Mani & Kay (105) using circular dichroism. They also found interaction between creatine kinase and myosin, heavy meromyosin, and subfragment one. Neither Botts et al nor Mani & Kay found an interaction of creatine kinase with the rod portion of myosin. The evidence indicates that creatine kinase is bound to the M-line of myofibrils, perhaps

through binding to the 165,000-dalton M-line protein, and also to subfragment one of heavy meromyosin, in the vicinity of the ATPase.

FUNCTIONAL STUDIES OF MYOFIBRILLAR CREATINE KINASE

Saks et al (106) studied the kinetics of myofibrillar creatine kinase—the MM isozyme. In contrast to mitochondrial creatine kinase, the kinetics of the myofibrillar isozyme at the concentrations of the substrates normally found in cells favor the synthesis of ATP from creatine phosphate and ADP.

Bessman et al (107) used ATP labeled with ^{32}P in the gamma position to study the functional relationship between myofibrillar creatine kinase and myosin ATPase. Myofibrils were incubated with creatine phosphate and ATP. The specific activity of the inorganic phosphate formed by the myofibrillar ATPase was compared to the mean specific activity of ATP over the incubation period. When the reaction was carried out in the presence of 1.6 mM ATP and 1.6 mM creatine phosphate the specific activity of the inorganic phosphate was less than the mean specific activity of the ATP, indicating that ATP regenerated from creatine phosphate had preferred access to the myosin ATPase. During a one-minute incubation, 30% of the inorganic phosphate came from creatine phosphate.

Further proof of the reliance of myofibrils on creatine phosphate to provide ATP for normal contraction was obtained by studying the contractile response of glycerinated muscle fibers to ATP and creatine phosphate (108, 109). In the presence of 250 μM ADP, the physiologic concentration of creatine phosphate (10 mM) produced faster and stronger contraction and faster and more complete relaxation than ATP at concentrations up to 10 mM. The apparent K_m for ADP added to fibers preincubated with 10 mM creatine phosphate was 1.2 mM. If the fibers were preincubated with ADP and contraction was initiated by the addition of 10 mM creatine phosphate, the apparent K_m of ADP for contraction was 0.076 mM. The apparent K_m of ADP for ATP synthesis by soluble creatine kinase was more than twice this value (0.15 mM), further indicating that when myofibrillar creatine kinase is compartmented and is associated with the myofibrillar ATPase, the juxtaposition of the two enzymes allows for more efficient provision of ATP by rephosphorylation of ADP than can occur in solution. These experiments were done in the presence of Ap5A, an inhibitor of adenylate kinase, to reduce any effects this enzyme would have on nucleotide concentrations. A slight difference in the diffusion coefficients of creatine phosphate and ATP has been suggested to play some role in explaining the greater efficiency of creatine phosphate and ADP than ATP alone in providing the energy for contraction, but this is not a complete explanation. The observation that 4 μM ADP and 10 mM creatine phosphate gives a greater

contraction than 100 μM ATP, a gradient that should overcome any differences in the diffusion coefficients, suggests that the enzymes are functionally linked (108, 109).

PROTEIN AND LIPID SYNTHESIS

Kleine (110) showed in 1965 that creatine kinase was associated with the microsomal fraction isolated from brain, skeletal muscle, and heart. Baba et al (111) showed that the MM isozyme was bound to microsomes in heart and skeletal muscle. Oganro et al (112) also described the presence of creatine kinase in the microsomal fraction of guinea pig heart.

We have shown that inhibition of creatine kinase with 2,4-fluorodinitrobenzene (FDNB) results in a parallel inhibition of lipid and protein synthesis in rat diaphragm (113, 114). FDNB at the concentrations used in diaphragm had minimal effects on protein and lipid synthesis in hepatocytes. At higher concentrations of FDNB there was a decrease in synthesis in hepatocytes, which correlated with a decrease in ATP levels, but ATP levels were not affected in diaphragm in FDNB concentrations that inhibited both lipid and protein synthesis. These experiments strongly suggest that the effects of FDNB on protein and lipid synthesis are due to inhibition of creatine kinase and not to some other effect of FDNB, since hepatocytes, which lack creatine kinase, and therefore must not utilize the creatine phosphate shuttle, are spared.

We have further shown that polysomes isolated from rat skeletal muscle contain endogenous creatine kinase (115). In the presence of creatine phosphate, ATP, and GTP, higher rates of protein synthesis are observed than with an ATP generating system of phosphoenolpyruvate and pyruvate kinase plus ATP and GTP. These data suggest that as with myofibrils, creatine phosphate provision of nucleoside triphosphates by rephosphorylation is more efficient than diffusion of nucleotides. When ^{33}P -creatine phosphate was incubated with the protein synthesizing system, the labeled phosphate was transferred to GTP. Presumably creatine phosphate regenerated ATP through the creatine kinase reaction. This ATP was used by nucleoside diphosphokinase, also present in the polysomes, to regenerate GTP. Creatine phosphate can therefore provide both ATP and GTP for protein synthesis in skeletal muscle.

Further confirmation of the role of the creatine phosphate shuttle in providing energy for protein synthesis is provided by work done by Bessman & Pal (116, 117). Creatine phosphate levels decrease in brain in a model of hepatic coma in which rats are injected with ammonium chloride. There is a smaller decrease in GTP and ATP levels. Accompanying the decrease in creatine phosphate is a decrease in protein synthesis in brains of animals treated with ammonium chloride. The inability of the mitochondria of ammonia-poisoned brain to regenerate creatine phosphate also prevents protein synthesis. Pre-

liminary experiments with FDNB show that protein synthesis in brain slices is even more sensitive to creatine kinase inhibition than in muscle tissue.

MAINTENANCE OF ION GRADIENTS

Sarcoplasmic Reticulum

Sarcoplasmic reticulum has been isolated in the microsomal fraction from muscle and heart, together with polypolysomes, making fractionation studies difficult to interpret. Baskin & Deamer (118) isolated microsomes from rabbit skeletal muscle and found creatine kinase activity. They proposed that the creatine kinase was associated with sarcoplasmic reticulum fragments and provided energy for calcium transport. Histochemical studies confirm the localization of creatine kinase in the sarcoplasmic reticulum, among other sites (111, 119).

Levitsky et al (120) isolated the MM isozyme from carefully fractionated sarcoplasmic reticulum. Creatine phosphate maintained a faster rate of calcium uptake by sarcoplasmic reticulum vesicles than an exogenous ATP-generating system of PEP and pyruvate kinase. This suggests that there is a functional coupling between ATP-dependent calcium transport and creatine kinase.

PLASMA MEMBRANE

Histochemical studies localized creatine kinase to the plasma membrane (119, 120) and Saks et al (121) found creatine kinase activity in isolated plasma membrane vesicles.

Several studies suggest that the creatine kinase associated with the plasma membrane functions to regenerate ATP for the sodium-potassium ATPase and ATP-dependent calcium transport. In 1960 Caldwell et al (122) showed that arginine phosphate injected into squid giant axon in which mitochondrial ATP production had been inhibited with cyanide maintained the sodium gradient. More recently Saks et al (121) have shown that the MM isozyme is associated with the plasma membrane in heart. Activity for the ATPase in a vesicle preparation is higher when ATP is regenerated by creatine phosphate rather than PEP and pyruvate kinase. Hydrolysis of creatine phosphate is inhibited by ouabain, an inhibitor of the sodium-potassium ATPase. Grosse et al (123) have shown that in the presence of creatine phosphate only 0.3 mM ATP is required to support maximal transport. These data again suggest functional coupling between creatine kinase and ATP requiring enzymes.

Spitzer et al (124) have shown, in similar experiments, that calcium transport by plasma membrane vesicles is faster when ATP is provided by creatine phosphate than the PEP, pyruvate kinase system.

Histochemical studies have identified creatine kinase in the nuclear mem-

brane (119) and Erashova et al (125) have found creatine kinase activity in nuclei isolated from heart. The function of creatine kinase in nuclei of heart and skeletal muscle cells has not been investigated. We found that RNA synthesis was not inhibited by the creatine kinase inhibitor FDNB (114). The creatine phosphate shuttle functions to regenerate ATP and in the case of nucleic acid synthesis, most of the nucleotides would be consumed and not available for rephosphorylation. Furthermore, the major free nucleotide products would be mononucleotides, which are inactive in the creatine kinase reaction. It is possible that some linkage with adenylate kinase, as in the "comparticle" (see below) might be active in the nucleus.

CREATINE KINASE IN OTHER TISSUES

Creatine kinase is found in other tissues than in muscle, heart, and brain. It seems generally to be associated with contractile proteins and cell movement. Creatine kinase is induced by estrogen in rat uterus (126, 127). The sequence of events suggests that it could play a role in providing the energy for protein synthesis. After estrogen stimulation creatine kinase activity increases for the next 6 hours. Thereafter there is an increase in protein synthesis by the uterus.

Creatine kinase is also found in leukocytes (128) and macrophages (129). In macrophages a large increase in the rate of turnover of creatine phosphate occurs with phagocytosis, suggesting that creatine phosphate provided the energy required.

AN INTEGRATED VIEW OF THE CREATINE PHOSPHATE SHUTTLE

The studies discussed have detailed the synthesis of creatine phosphate by mitochondria, and its use by ATP requiring reactions throughout the cell. The use of creatine phosphate by these reactions involves a close association of creatine kinase and the local ATPase which allows creatine phosphate to regenerate ATP at or very near the active site of the ATPase. This allows the effective concentration of ATP near the active site to be very high so that the reaction is not limited by the availability of energy. As a corollary of this theory, adenine nucleotides are considered to be compartmented at strategic sites with a high concentration of ATP only in the intramitochondrial space and of ADP at the peripheral ATPase.

The presence of creatine kinase, creatine, and creatine phosphate in high concentrations in only three tissues, heart, skeletal muscle, and brain, may provide a clue to the evolutionary origin of the creatine phosphate shuttle. Unique to skeletal muscle and heart is their contractile function. Creatine phosphate may have served as an energy buffer, for it is far more basic than

ATP and would remain electrostatically in the region of the acidic myosin. Release of an even more basic free creatine would tend to displace phospho-creatine providing more energy for further contraction. An advantage may have been conferred when creatine kinase became closely associated with the myosin ATPase. ATP would be more efficiently regenerated and could better support contractile work. Development of the mitochondrial creatine kinase compartment resulted in more efficient synthesis of creatine phosphate. The more rapid diffusion of creatine phosphate than ATP provided a more efficient energy supply. Creatine phosphate no longer was simply an energy buffer but evolved into the means of energy transport.

In parallel with these developments creatine kinase may have become associated with other ATPases. This is a necessary development to assure ATP provision in the presence of adenine nucleotide compartmentation.

Brain tissue presumably has no major variation in the demand for energy as does the muscle and would not require creatine to buffer this demand. Continued energy supply may be so crucial for brain that a creatine phosphate shuttle system developed not to buffer changes in energy demand but to ensure an adequate energy supply under extreme conditions such as hypoxia.

Our view of the creatine phosphate shuttle is that it serves two distinct functions in the heart, muscle, and brain. The first is to deliver creatine as the signal or stimulus of oxygen uptake mediated by respiratory control, and the second is to provide creatine phosphate, the form of energy moving from mitochondrion to utilization site.

Mainwood & Rakusan (130) presented a model of intracellular energy transport in which mitochondria cluster around capillaries and the creatine kinase reaction is a near-equilibrium reaction at all rates of ATP consumption. According to this model individual molecules of creatine phosphate do not diffuse from mitochondria to ATPases in the cytosol, rather the reaction is maintained in near-equilibrium throughout the cell. Deviations from equilibrium in a part of the cell are quickly corrected by distribution throughout the cell. Meyer et al (131) have termed this model "facilitated diffusion." Evidence suggests that the assumptions on which the model is based are not valid. The substrates of the creatine kinase reaction and the enzyme itself are compartmented, not distributed homogeneously throughout the cell. About half of the creatine kinase in muscle cells is bound to myofibrils and mitochondria. The state of the rest of the creatine kinase in the intact cell is not known. Based on evidence that creatine kinase is bound to myofibrils and in association with the ATP- and GTP-requiring reactions of protein synthesis and to the enzymes of lipid synthesis, as well as to the ATP-dependent ion pumps, creatine kinase may not be free in the cytosol. It may be bound near these and other ATPases, but released by the fractionation procedures making it appear to be a soluble enzyme.

The question of whether the creatine kinase reaction is in equilibrium has been addressed directly using ^{31}P -NMR techniques. As would be expected the reaction appears to be in equilibrium in resting skeletal muscle (132). However, in contracting skeletal muscle and heart, the reaction does not appear to be in equilibrium. Gadian et al (133) concluded that the creatine kinase reaction was in equilibrium in resting muscle if a correction was made for a presumed underestimation of flux from ATP to creatine phosphate. In contracting muscle the reaction was not in equilibrium. Fossel et al (134) found that in contracting heart creatine phosphate and ATP levels decreased by 15% between diastole and systole. This is compatible neither with the energetics of the process nor the direct results of any other investigators. Mathews et al (135) found that the rate of ATP synthesis in perfused rat heart is five times less than the flux through the creatine kinase reaction and concluded that the creatine kinase reaction could be maintained near equilibrium. They point out, however, that the oxygen consumption, and thus ATP synthesis, in a working heart is five times that of the Langendorf preparation used in their experiment (136). It is likely that the reaction is not in equilibrium in *in vivo* working heart and contracting muscle *in vivo*. Since creatine phosphate can only be converted to ATP, flux should be equal for creatine phosphate and ATP. The discrepancies above indicate the serious technical problems that beset NMR measurements to date.

STUDIES OF THE CREATINE PHOSPHATE SHUTTLE USING CREATINE ANALOGS

Several analogs of creatine have been used to study the creatine phosphate shuttle in intact tissue. The analogs were fed to animals (mice, rats, or chickens) over a period of weeks. Creatine uptake was inhibited by the analogs, which are themselves taken up and phosphorylated, resulting in a depletion of creatine phosphate and creatine and an accumulation of the analog and its phosphorylated derivative (137). The analogs and the phospho-derivatives are poor substrates for creatine kinase (138).

Shields et al (139) found that type II fibers (white) from rat gastrocnemius muscle were smaller in animals treated with B-guanidinopropionic acid (B-GPA). Petrofsky & Fitch (140) found that the plantaris muscle in rats treated with B-GPA was 30% lighter (wet weight) than in control animals. There was no difference in the soleus muscle. Laskowski et al (141) studied chickens fed with B-GPA and found growth retardation and weakness. Microscopically there was loss of thin and thick filaments, disruption of the Z band, and dilatation of mitochondria and sarcoplasmic reticulum.

All investigators have found marked depletion of creatine and creatine phosphate, usually 80–90%, and accumulation of the analogs. There is also a depletion in ATP levels of 40–80%. Shields et al (139) also described a

decrease in creatine kinase activity. Petrofsky & Fitch (140) found little difference in the contractile characteristics of plantaris muscle from creatine depleted and normal rats. There were marked differences in the soleus muscle, however: a decrease in the rate and amplitude of contraction and in the rate of relaxation. Mainwood et al (142, 143) found decreased response to electrical stimulation in diaphragm from rats treated with B-GPA. Diaphragms from treated animals would contract only 4 times compared to 15 times for the control. They also found a decreased maximum tension and a decrease in the rate of tension development and relaxation.

Fitch et al (144) described a prolongation of isometric contraction in soleus, but not plantaris muscle from treated rats. Annesley & Walker (145) found that treatment with cyclocreatine led to a delay in the development of rigor.

Although chickens are severely affected by creatine depletion, rats and mice are grossly relatively little affected. An increase in the flux of the creatine phosphate that remains may be sufficient to supply energy for animals under normal, unstressed circumstances. We must again consider the possible major difference between measured concentrations and biological activity of creatine and creatine phosphate.

DEVELOPMENT OF THE CREATINE PHOSPHATE SHUTTLE

Only small concentrations of creatine and creatine phosphate are necessary to transfer energy under ordinary circumstances. The remainder of the creatine phosphate may be a reservoir, bound to myosin. The creatine phosphate shuttle does not seem to exist in fetal animals' heart, yet the heart contracts, albeit slowly. The creatine phosphate shuttle develops around the time of birth, both the enzymes and the substrates increasing roughly in parallel to the activity of the heart.

Hall & DeLuca (146) have shown that there is no mitochondrial creatine kinase in neonatal rat hearts, so there could be no compartmented synthesis of creatine phosphate coupled to oxidative phosphorylation. Ingwall and coworkers (147, 148) have shown that from about gestational day 14 through birth there is an 8–9-fold increase in total creatine kinase activity and a 13–15-fold increase in the concentration of total creatine (creatine phosphate plus creatine phosphate). Roberts & Bessman (149) have shown that there is about a 7-fold increase in the concentration of creatine phosphate from gestation day 14 to the newborn period.

Pette (149a) has conducted a series of studies on muscle function and enzyme behavior that relate enzyme composition to type of stimulation. The whole muscle fibers that are fast contain relatively few mitochondria and are primarily

glycolytic whereas the slow, red fibers contain large amounts of mitochondria, and are primarily oxidative.

When the fast muscles are continuously stimulated at the low rates normal for slow muscles, or when the innervation of fast muscles is changed to the innervation of slow muscles there are marked changes in the enzyme content of the muscle fibers. The enzymes of the glycolytic pathway, except for hexokinase, all fall, and the enzymes of the oxidative pathways increase, with the increase of mitochondria. Hexokinase also increases, which has led Pette (149a) to conclude that hexokinase activity is more a function of respiratory control than of glycolysis (cf the theory of insulin action above). Creatine kinase total activity drops by 30–40%.

There is no information on the isozyme distribution as affected by the change in stimulation, but if the pattern shown by all of the glycolytic and oxidative enzymes tested also applies to creatine kinase one would expect a large increase in mitochondrial creatine kinase and a drop in the MM function. Studies of the isozymal distribution under these circumstances might give very interesting insights into the actual quantity of “free” creatine kinase, for that portion should be related to the soluble glycolytic system. Gonner, Loike et al (149b) have shown a relation between differentiation of macrophages in culture and the appearance of creatine kinase (BB) and creatine phosphate.

ADENYLATE KINASE AND THE CREATINE PHOSPHATE SHUTTLE

Evidence has been obtained for a direct role of adenylate kinase in both the mitochondrial and myofibrillar ends of the shuttle.

Yang et al (88), in their early tracer studies of the mitochondrial synthesis of creatine phosphate from nascent ATP, found that specific activities of creatine phosphate measured in 5 and 10 sec incubations showed that about one-third of the phosphoryl group of creatine phosphate phosphorus could have come directly from oxidative phosphorylation and the rest must have come from other sources of ATP including the gamma ^{31}P -labeled ATP added as tracer. They concluded that two adenylate kinase molecules might be interposed between the oxidative phosphorylation site (translocase) and the mitochondrial creatine kinase. (Figure 3). The two adenylate kinase molecules acting in sequence in opposite directions on a common pool of ADP could produce a distribution of isotope which would match the experimental data. A particle or compartment containing two molecules of adenylate kinase and one of creatine kinase all clustered in a tetrahedral pattern (Fig. 4) around the translocase was designated the “comparticle” (150).

In studies of the histochemical distribution of ATPase in myofibrils using

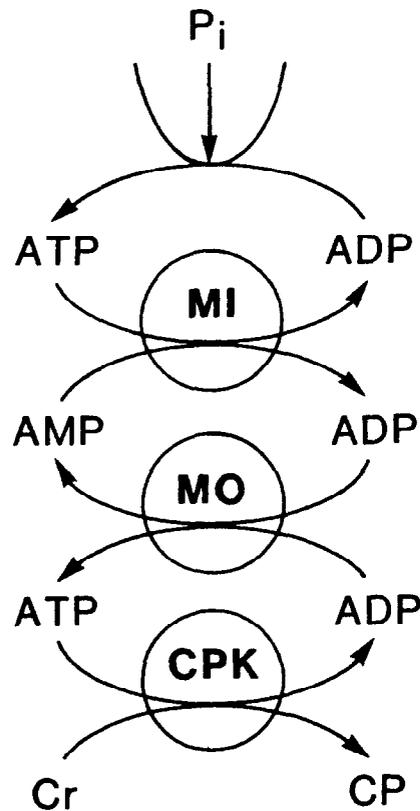


Figure 3 The sequential operation of two adenylate kinase enzymes, designated MI and MO, which tend to maintain direction and appropriate concentration for the conversion of creatine to creatine phosphate in the mitochondrion.

lead precipitation of phosphate liberated, Tice & Barnett (151) showed that both ATP and, to a lesser extent, ADP, caused the formation of free phosphate throughout the M-band as well as the Z-line. They concluded that adenylate kinase was located near the ATPase activities. Savabi et al (152) observed that glycerinated rabbit muscle fibers that contracted on the addition of ATP would relax spontaneously to a great extent without the addition of relaxing solutions. This relaxation could be abolished by the addition of P^1, P^5 -di (adenosine 5')pentaphosphate Ap5A a specific inhibitor of adenylate kinase. Furthermore, in the presence of Ap5A it was possible to show that any concentration of creatine phosphate with 200 μ M ADP was more effective in causing both contraction and relaxation than an equivalent concentration of ATP. This suggests that the "comparticle" structure may also reside in the myofibril. The first enzymes to contact creatine phosphate would be a pair of adenylate kinase molecules followed by myosin ATPase (Figure 5). There would be an ADP pool in the central region as in the mitochondrial "comparticle" (Figure 6).

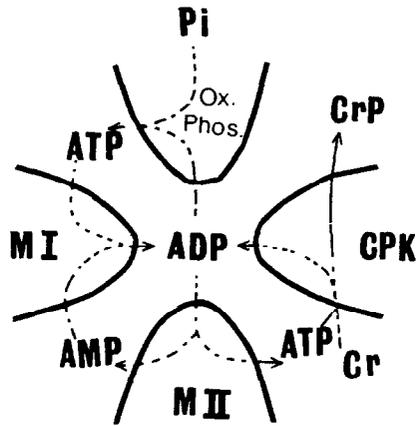


Figure 4 The arrangement of the three enzymes, the two adenylate kinase molecules and one creatine kinase around the site of oxidative phosphorylation to produce a common pool of ADP and sites of entry for ATP, creatine-creatine phosphate, and AMP.

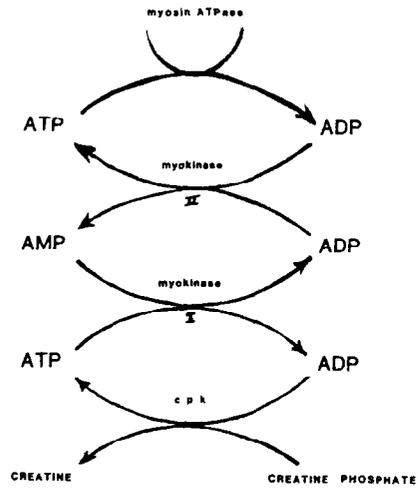


Figure 5 The sequential arrangement of adenylate kinase molecules to maintain substrate flow between creatine kinase and myosin ATPase.

POSSIBLE PATHOLOGICAL SIGNIFICANCE OF THE CREATINE PHOSPHATE SHUTTLE

Two general areas of concern relevant to the creatine phosphate shuttle and disease are, first, those diseases in which there would be a deficiency of creatine kinase, and second, those diseases that might arise from a deficiency of creatine.

In the first case it was shown in our laboratory (153) that as muscular dystrophy develops in the chicken model there is a progressive fall in mitochondrial creatine kinase activity. DeLuca's laboratory reports (154) an extensive analysis of this phenomenon, showing a strong correlation between function and the ratio of mitochondrial creatine kinase and succinate-iodo-tetrazolium reductase, a mitochondrial marker. The dystrophic chicks had a greater resistance to loss of creatine kinase from mitochondria caused by phosphate. The relevance of these findings to the human disease is discussed and several parallels are drawn. It is possible from their data to propose at least two forms of human muscular dystrophy—one deficient in the myofibrillar enzyme and one deficient in the mitochondrial isozyme. It would be necessary in any assay, in view of the foregoing evidence, to test for these possible lesions by the forward synthetic reaction in the mitochondria and the backward (ATP synthesis) reaction in myofibrils.

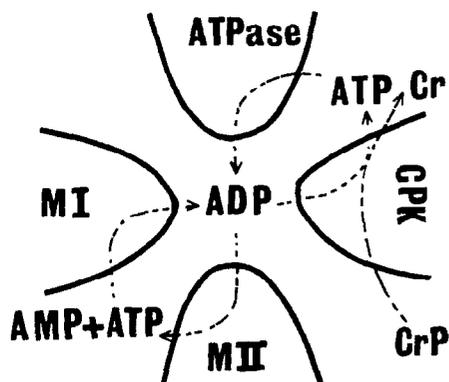


Figure 6 A tetrahedral arrangement of adenylate kinase molecules with creatine kinase around the myosin ATPase, in such a way that there is a common pool of ADP and entrance sites for ATP, AMP, creatine, and creatine phosphate. In this diagram the principal pathway for energy to be made available for the complex is from creatine phosphate to ATP.

PHOSPHATE DEPLETION

Work by Brautbar et al (155, 156) suggests that the heart and muscle disease that result from phosphate depletion may be a result of a defect in the creatine phosphate shuttle. Rats maintained on a phosphate deficient diet became deficient in myocardial inorganic phosphate, creatine phosphate, and adenine nucleotides. Total and mitochondrial creatine kinase levels were also decreased in both heart and skeletal muscle as was the mitochondrial respiratory rate. A decreased ability to produce, transport, and use creatine phosphate may explain the myopathy and cardiomyopathy of phosphate depletion.

The source of tissue creatine is a complex series of reactions involving, in the human, the liver, kidney, and pancreas. The liver is of particular importance, for the precursors of creatine, arginine and glycine, are synthesized there. One could expect serious curtailment of supply of creatine in liver disease. Perhaps this is one mechanism by which some beneficial effects have been reported for use of large amounts of arginine in treatment of hepatic coma (157). It has been estimated that at least 87 g of arginine are synthesized and broken down daily by the normal adult (158).

The liver is also the site of methylation of the guanidoacetic acid formed from glycine and arginine in the kidney. Since the methylation requires methionine, deficiency of this amino acid might be responsible for weakness, and, in the brain, of functional problems due to creatine deficiency. Mental retardation could be related to deficiency of creatine in utero caused by any lesion of the arginine cycle (improperly called the urea cycle). The fetus would make little arginine and would receive less than normal amounts from the heterozygous mother (159). This could be the basis of mental retardation associated with inability to synthesize glycine as well.

Gyrate atrophy of the retina has been shown to be associated with ornithinuria (160-162). It is possible that creatine synthesis from arginine is impaired

and reveals itself as a failure in energy generation in the retina. The arginine cycle is a function of the prenatal brain (158) and probably the retina as well.

EXERCISE AND MUSCLE GROWTH

The muscle hypertrophy of exercise is accompanied by the increased activity of the shuttle caused by muscle contraction. The delivery of creatine phosphate to protein synthesizing sites will be increased by exercise and at least 70% of protein synthesis is dependent on this source of energy (113–115).

Hypertensive cardiac hypertrophy could occur by a similar mechanism. The increased vascular resistance in hypertension would stimulate increased cardiac contraction and this increased contractile activity would stimulate protein synthesis resulting in the enlarged myocardium found in chronic hypertension.

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