Evidence for Compartmentalized Adenylate Kinase Catalysis Serving a High Energy Phosphoryl Transfer Function in Rat Skeletal Muscle*

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The first characterization of the kinetics and subcellular compartmentation of adenylate kinase activity in intact muscle has been accomplished using rat diaphragm equilibrated with [14O]water. Rates of adenylate kinase-catalyzed phosphoryl transfer were measured by appearance of [14O]-labeled β-phosphoryls in ADP and ATP resulting from the transfer to AMP of newly synthesized [14O]-labeled γ-ATP. Unique features of adenylate kinase catalysis were uncovered in the intact cell not predictable from cell-free experiments. This enzyme activity, which in non-contracting muscle is limited to 1/1000 of the estimated Vmax (cell free) apparently because of restricted ADP availability, is localized in subcellular compartments that increase in size and/or number with contractile frequency. Contraction also causes frequency-dependent increments in adenylate kinase velocity (22-fold at 4 Hz) as does oxygen deprivation (35-fold). These enhanced rates of adenylate kinase activity, equivalent to processing all the cellular ATP and ADP in approximately 1 min, occur when levels of ATP, ADP, and AMP are maintained near their basal steady state. These characteristics of the dynamics of adenylate kinase catalysis in the intact cell demonstrate that rapid rates of AMP production from ADP are balanced by equally rapid rates of AMP phosphorylation with no net synthesis or accumulation of any adenine nucleotide. This rapid processing of nucleotide phosphoryls conforms to a proposed scheme whereby the adenylate kinase system provides a unique function of transferring, as β-ADP, high energy phosphoryls generated by glycolytic metabolism to ATP-utilizing components in muscle.

Adenylate kinase (ATP:AMP phosphotransferase, EC 2.7.4.3) catalyzes the readily reversible transphosphorylation of AMP using γ-ATP as the preferred phosphoryl donor. The enzyme is found in almost all cell types, exists as isoforms, and is especially abundant in cells having high rates of ATP synthesis and utilization such as skeletal muscle (1, 2). In skeletal muscle adenylate kinase is physically associated with mitochondria and with myofibrils, subcellular structures that are involved in energy production and utilization, respectively. This apparently discrete localization indicates that β-phosphoryl metabolism by adenylate kinase isozymes is likely to be compartmentalized (3, 4).

At present, adenylate kinase activity is thought to be at or near equilibrium in the cell and regulated only by the availability of AMP or ADP, the concentrations of which can increase when primary energy-providing metabolic pathways become compromised. The intracellular concentration of free magnesium has also been suggested to be a regulatory determinant of the velocity of this enzyme (5), although the physiological circumstance underlying such a mechanism remains obscure. The constancy of the cellular energy charge during ATP utilization prompted the suggestion that adenylate kinase functions simply to equilibrate the stores of adenine nucleotides and thereby make available the energy inherent in the adenine nucleotide β-phosphoryl (2). Others, however, have proposed that adenylate kinase is an integral part of a high energy phosphoryl transfer system operating in concert with creatine kinase to transfer energy-rich phosphoryls at mitochondrial and myofibrillar sites (6). The latter concept is supported by the demonstration that the adenylate kinase inhibitor, diadenosine pentaphosphate, diminishes the rates of contraction and relaxation in permeabilized muscle fibers (6). It is also consistent with the observation that the phosphoryl of creatine phosphate, when synthesized by isolated mitochondria, is derived in part from the β-phosphoryl of ATP(7, 8). In addition, ADP generated in the mitochondrial intermembranous space by adenylate kinase or creatine kinase has been reported to be more effective at inducing state 3 respiration in isolated mitochondria than ADP added directly to mitochondria (9). Recently, evidence was presented consistent with an involvement of adenylate kinase as a provider of metabolic energy in bovine sperm flagellum (10).

Nevertheless, the proposals regarding the involvement of adenylate kinase in high energy phosphate metabolism and/or transfer remain speculative for lack of information about the rates of adenylate kinase catalysis that occur in cells and the physiological or metabolic circumstances that govern its participation.

Adenylate kinase catalysis has been observed to occur in intact diaphragm muscle by the appearance of 32P in β-ADP and β-ATP in tissues incubated in the presence of [32P]-orthophosphate (9). However, the very slow rate of orthophosphate transport into cells, as well as the likelihood of its non-uniform incorporation into metabolic compartments, renders kinetic analysis by this experimental approach difficult.
to interpret and/or incomplete. Meaningful examination of adenine nucleotide metabolism in skeletal muscle and the metabolic flux of nucleotides through the adenylate kinase pathway undoubtedly requires an analytical procedure that preserves the integrity of the complex compartmental relationships existing in the intact cell. Some information about high energy phosphate metabolism in intact muscle has been obtained by NMR technology which, however, has the constraint of limited sensitivity due to protein binding of nucleotides as well as limitations in determining metabolic changes associated with the contractile process (11, 12). We have found that hydrolytic cleavages and the transfer of phosphoryls can be readily monitored in intact cells by simply incubating the tissue in medium enriched in $^{18O}$ water and determining the rate of appearance of $^{18O}$ in the phosphoforms of interest. Because there is apparently no barrier for the rapid exchange of medium and cellular water, the $^{18O}$ water equilibrates in isolated cells and thin tissues rapidly (within about 1 s; 13). Enzyme-catalyzed hydrolytic cleavages of $\gamma$-ATP and $\gamma$-GTP result in stoichiometric $^{18O}$ incorporation into cellular orthophosphate, and esterification of ADP to ATP with this $^{18O}$-containing orthophosphate results in isotopic labeling of the $\gamma$-phosphoryl of ATP (i.e. $[\gamma$-$^{18O}]$ ATP). The rate of turnover of the $\gamma$-phosphoryl of ATP is relatively fast and equilibration of the $\gamma$-phosphoryl oxygens with $^{18O}$ occurs rapidly (14). Adenylate kinase-catalyzed transfer of a $[\gamma$-$^{18O}]$ATP to AMP thus results in the appearance of an $^{18O}$-labeled phosphate in $\beta$-ADP. The resultant $[\beta$-$^{18O}]$ADP can be phosphorylated to $[\beta$-$^{18O}]$ATP or used with another molecule of ADP to reform a molecule of AMP and a molecule of $[\gamma$-$^{18O}]$ATP by adenylate kinase catalysis. AMP generated by way of metabolic pathways other than adenylate kinase (i.e. hydrolysis of CAMP or pyrophosphohydrolytic cleavage of ATP) can be identified by the presence of $^{18O}$ in the phosphoryl of AMP and, therefore, in the $\alpha$-phosphoryl of ADP or ATP, which is a consequence of these reactions but not of adenylate kinase catalysis. From these characteristics of $^{18O}$ labeling of adenine nucleotide phosphoryl monitoring rates of adenylate kinase catalysis has been possible under nearly physiological conditions in intact skeletal muscle at rest, during contraction, and in different definable metabolic states. Componental characteristics of adenylate kinase substrates and products are also revealed when the phosphoryls of only a fraction of the total cellular content of a nucleotide metabole are labeled when $^{18O}$ incorporation to equilibrium is attained.

The results of these experiments provide the first direct measurement of adenylate kinase velocities in intact muscle cells and include information on 1) the basal rates of adenine nucleotide metabolic flux through adenylate kinase and the effect of contraction on these rates, 2) the change in the fractions of the total cellular ADP and ATP that actively participate in adenylate kinase-catalyzed metabolism and between contractile states of the muscle, 3) the effect of compromising oxidative metabolism on $\beta$-phosphoryl metabolic flux, 4) an estimate of what the metabolically effective concentration of ADP is in cellular locales where adenylate kinase catalysis takes place, and 5) the metabolic status of protein-bound and unbound species of cellular ADP.

A major conclusion from these results is that adenylate kinase catalysis occurs in localized subcellular compartments in conjunction with increased rates of high energy phosphate utilization occurring with contraction and when the rate of glycolytic metabolism increases under anaerobic conditions to sustain near maximal high energy phosphate status. The results obtained are not compatible with adenylate kinase functioning to partially compensate for declining levels of cellular high energy phosphates by salvaging ATP from ac-cumulating ADP. Instead the data support the concept that this enzyme system functions to transfer high energy phosphoryls from a primary energy-providing metabolic pathway to ATP-utilizing components of muscle.

**EXPERIMENTAL PROCEDURES**

**Tissue Preparation and Incubation** The rat diaphragm muscle, with ribs attached, was prepared as previously described (15). Male Sprague-Dawley rats (Bio-Lab) weighing 150-200 g were anesthetized with sodium pentobarbital (50 mg) and the diaphragm muscle was suspended in a chamber and placed in a buffer containing 135 mM NaCl, 4 mM KCl, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 1 mM NaHPO$_4$, 20 mM NaHCO$_3$, and 10 mM glucose, pH 7.4. Intercostal muscle and non-muscle tissue were removed as completely as possible without disrupting the diaphragm muscle while incubating in the buffer. The preparation was attached to a Lucite holder by ligatures to the ribs and incubated at 24°C in a Lucite chamber containing 12 ml of the above medium that was continuously bubbled with O$_2$/CO$_2$ (95/5%). In some experiments oxygen was purged from the medium by bubbling with nitrogen prior to and during the experiment. The tissue was equilibrated for 10 min in the chamber while the muscle was stimulated to twitch at a frequency of 0.5 Hz by direct electrical stimulation (10 ms duration, 10 V) through platinum electrodes (2 x 2.5 cm) placed transversely 0.5 cm from the muscle. The tissue was then transferred to a chamber containing medium identical to that present in the first chamber except that it was enriched with 30-45 atom % excess in $[^{18}]$O-water (Bio-Lab Co.). Incubation in $[^{18}]$O-water containing medium was continued for various times with or without stimulation. Incubations were terminated by rapid transfer of the tissue into liquid nitrogen. The tissues were stored for no more than 12 h on dry ice prior to processing.

**Extraction of Tissue Nucleotides**—The frozen tissue was allowed to warm to 0°C, and the ribs and central tendon were dissected and discarded. The diaphragm muscle tissue was ground to a fine powder in a liquid nitrogen-cooled porcelain mortar. In experiments where the total acid-extractable nucleotides were analyzed, the frozen powdered tissue was placed in a tube containing 1 ml of frozen 3 M perchloric acid and allowed to warm to −10°C, the melting point of this solution, allowing the still frozen tissue to become submerged in the liquefied acid. The temperature of perchloric acid was then raised to 0°C and diluted to 0.8 M with ice-cold 5 mM EGTA and the protein was removed by centrifugation. The supernatant from the acid extract was neutralized with 2 M KHC$_2$O$_3$, the KClO$_4$ precipitate was removed by centrifugation, and the supernatant was evaporated to dryness at reduced pressure.

Extraction of adenine nucleotide phosphoryl metabolism in adenine nucleotides that are tightly bound to myofibrils powdered tissue was extracted with 5 ml of a solution containing 10 mM MOPS, pH 7.0, 100 mM KCl, 10 mM EGTA, 2 mM MgCl$_2$, 0.5% Triton X-100, and 1 mg/ml apyrase for 15 min at 0°C with frequent mixing followed by centrifugation at 1000 x g for 10 min. The pellet was then washed by centrifugation in the same solution without Triton X-100, and the bound nucleotides in the resulting myofibril pellet were extracted with perchloric acid and neutralized as described above.

The supernatant from the above extraction procedure was unsuitable for examining the metabolic properties of free or loosely bound nucleotide phosphoryls. To accomplish this analysis frozen, powdered diaphragm muscle was suspended in 3 ml of a solution of 10 mM MOPS, pH 7.0, 100 mM KCl, 10 mM EGTA, 2 mM MgCl$_2$, and 70% ethanol at −20°C and extracted for 10 min with occasional mixing. The samples were then centrifuged at 8000 x g for 5 min. The pellet was suspended in 1 ml of the MOPS-buffered solution and the extraction procedure repeated. The supernatants from the two extractions were combined and any soluble precipitin (95%) that was present was precipitated by the addition of 1 ml of 3 M perchloric acid. The first and second extractions contained 28 and 10%, respectively, of the total cellular ADP.

**Purification of 5'-Nucleotides and $^{18}$O Analysis of Phosphoryls**—The methods for purification of cellular 5'-nucleotides, creatine phosphate, and orthophosphate, and the analytical techniques used to
Adenine Nucleotide β-Phosphoryl Transfer in Skeletal Muscle

determine the $^{18}O$ in these phosphoryls have been described in detail elsewhere (13). Briefly, the cellular 5'-nucleotides were initially purified on phenylboronate agarose resin (PBA-60, Amicon) followed by separation using anion exchange HPLC (AG MP-X1, Bio-Rad). The γ-phosphoryl of the purified ATP was transferred to glycerol using glycerokinase while the β-phosphoryl of ADP and ATP were transferred to glycerol by coupling adenylate kinase and glycero kinase. The phosphoryl of creatine phosphate was transferred to glycerol using creatine kinase and glycero kinase. None of the above phosphoryl transfers occurs to the $^{18}O$ enrichment of the phosphoryl transferred. The $^{18}O$ content of the α-phosphoryl of adenine nucleoside monophosphates as well as the monophosphates deriving from the nucleoside di- and triphosphates were analyzed as glycerol 3-phosphate following chemical degradation of the nucleoside monophosphate. All the samples analyzed by mass spectrometry were converted to their trimethylsilyl derivatives using bis(trimethylsilyl)trifluoroacetamide and the $^{18}O$ enrichment was determined using a Hewlett-Packard 5970B gas chromatograph-mass spectrometer operated in the selective ion-monitoring mode. In all experiments tissue samples, which were incubated in $[^{18}O]$water, were carried through the procedures for determining the natural abundances of oxygen isotopes. In the analysis of glycerol 3-phosphate the masses (m/z) 357, 359, 361, and 363 were monitored and correspond to $^{18}O$, $^{18}O_2$, $^{18}O_3$, and $^{18}O_4$, respectively. The bis (trimethylsilyl)trifluoroacetamide derivative of orthophosphate yielded mass ions (m/z) 299, 301, 303, 305, and 307 corresponding to species of $[^{18}O]$orthophosphate containing 0, 1, 2, 3, and 4 atoms of $^{18}O$.

Computer Simulation of $^{18}O$ Appearance in β-ADP and β-ATP—
The kinetics of adenine nucleotide β-phosphoryl labeling with $^{18}O$ were simulated using the Stella modeling program (High Performance Systems, Inc., Lymo, NH) on a Macintosh II computer. Given an observed time course of $^{18}O$ labeling of γ-ATP, the model computes theoretical time courses of $^{18}O$ incorporation into β-ADP and β-ATP. The computation depends on the fractions of ADP and ATP that have β-phosphoryls which are metabolically active. These two metabolic pool sizes can either be experimentally measured and the values supplied to the model, or they can be parameters of the model to be estimated when the theoretical time courses of $^{18}O$ incorporation into β-ADP and β-ATP are fitted to data. The model is based on two rates of phosphoryl conversion. F$_1$ is the rate at which the γ-phosphoryl of ATP is transferred to an AMP molecule by adenylate kinase to form a β-phosphoryl of ADP. F$_2$ is the rate at which the β-phosphoryl of ADP is converted to a β-phosphoryl of ATP by phosphorylation of ADP to ATP. Two assumptions are made to balance these two fluxes in order to conform with the observed steady state condition. First, it is assumed that the rate of conversion of β-ATP to β-ADP is equal to F$_2$; second, it is assumed that the rate of disappearance of β-ADP to forms other than β-ATP is equal to F$_1$. Finally, it is assumed that the metabolites in the β-phosphoryl metabolic compartment are well mixed.

The significance of $F_1$, $F_2$, and the steady state assumptions are as follows. The steady state assumption that sets the rate of disappearance of β-ADP to forms other than β-ATP equal to $F_1$ means that the model considers only two fates of β-ADP: 1) β-ADP can be converted to β-ATP or 2) β-ADP can be removed by adenylate kinase to form γ-ATP. Specifically, it is assumed that $5'$-nucleotidase activity is negligible in comparison to adenylate kinase activity, which is a reasonable assumption. $F_2$ is the combined velocity of all reactions that convert ADP to ATP including oxidative phosphorylation and substrate level phosphorylation, creatine kinase, and adenylate kinase. Therefore $F_2$ is greater than $F_1$ since adenylate kinase activity ($F_1$) contributes minimally to the ADP to ATP conversion. The assumption that the rate of the reverse reaction, ATP to ADP, is also equal to $F_2$ implies that the only fate of ATP is to become ADP. Specifically, it is assumed that the rates of adenyl cyclase, pyrophosphohydrolase, and other non-adenylate kinase means of forming AMP are negligible in comparison to the rate at which ATP is converted to ADP. The rate at which ATP is converted to ADP should not be equated with the sum of ATPase velocities since it also includes kinase activities such as adenylate kinase, creatine kinase, and protein kinases. A value for $F_2$ can be supplied to the model from measurements of γ-ATP labeling or, alternatively, $F_2$ can be a free parameter to be estimated when the model predictions are fitted to data.

RESULTS

Theoretical Considerations of $^{18}O$ Incorporation into Metabolic Intermediates—Enzyme-catalyzed hydrolysis of the $\gamma$-β bond of nucleoside triphosphates results in the insertion of an atom of $^{18}O$ from $[^{18}O]$water into the orthophosphate product (Reaction 1; 16). With repeated cycles of nucleoside triphosphate synthesis and hydrolysis cellular orthophosphate containing from zero to four atoms of $^{18}O$ is formed. Phosphorylation of ADP by oxidative phosphorylation or substrate level phosphorylation results in the random loss of one oxygen atom from orthophosphate and therefore the γ-phosphoryl of ATP can be labeled with zero to three atoms of $^{18}O$ (designated $^{18}O_0$, $^{18}O_1$, $^{18}O_2$, and $^{18}O_3$; Reaction 2). These properties of the synthetic and hydrolytic reactions of ATP (and GTP) have been used to determine high energy phosphate utilization rates from the incorporation of $^{18}O$ into phosphoryls of metabolic intermediates (14).

\[
\text{ADP} - P - O + H_2^{18}O \rightarrow \text{ADP} - O - P - O (\text{Reaction 1})
\]

\[
\text{ADP} + O - P - O - \text{ADP} - P - O (\text{Reaction 2})
\]

\[
\text{ADP} - P - O + AMP - AMP - O + AMP - P - O - O (\text{Reaction 3})
\]

From our current understanding of nucleoside polynucleotide metabolism the appearance of an $^{18}O$-labeled β-phosphoryl in ADP can arise only upon adenylate kinase-catalyzed transfer of an $^{18}O$-labeled γ-phosphoryl from ATP to AMP (Reaction 3). Enzymic transfer of this $^{18}O$-labeled γ-phosphoryl to AMP, forming $[^{18}O]$ADP, occurs without the loss or gain of oxygen atoms from the γ-phosphoryl (17). Furthermore, upon phosphorylation of $[^{18}O]$ADP to $[^{18}O]$ATP the oxygens in the β-phosphoryl position are retained. Adenylate kinase, therefore, generates $[^{18}O]$ADP for ATP with the identical $^{18}O$ enrichment as the γ-phosphoryl from the donor pool(s) of ATP. The rate of appearance of $[^{18}O]$phosphoryls is, therefore, a measure of the velocity of adenylate kinase and can be determined in the intact cell by substituting a fraction of the $[^{18}O]$water in the incubation medium with $[^{18}O]$water.

Quantitative information about the fraction of a cellular nucleotide metabolically active can be obtained by determining the proportion of the total cellular nucleotide that becomes labeled when the phosphoryl of interest achieves saturation. Saturation labeling of phosphoryl oxygens occurs when the maximum possible number of $^{18}O$ atoms becomes incorporated as a result of the aforementioned metabolic recycling. This end-state distribution of $^{18}O$-containing phosphoryls is a function of the $[^{18}O]$water enrichment and can be predicted by a binomial distribution function (14). The fraction of nucleotides with phosphoryls undergoing active metabolism ($F_{\text{met}}$) is equal to the ratio of the experimentally determined weighted sum of $^{18}O_0$, $^{18}O_1$, $^{18}O_2$, and $^{18}O_3$ species to the value of this sum for a theoretical pool that is 100% metabolic. (See Ref. 14 for a complete discussion of the factors affecting $^{18}O$ incorporation into nucleotide phosphoryl in intact tissues.) $^{18}O$ Appearance in the γ-Phosphoryl of ATP—Factors influencing the rate of γ-ATP labeling with $^{18}O$ bear directly on
assessing the kinetics of \( \beta \)-phosphoryl transfer by analysis of \( ^{18} \text{O} \) appearance. The rates of appearance of each of the \( ^{18} \text{O} \)-labeled species of \( \gamma \)-ATP (i.e. with 1, 2, or 3 atoms of \( ^{18} \text{O} \)) are relevant to measurement of high energy phosphate utilization rates which is the subject of another report. For the purpose of monitoring rates of adenylate kinase-catalyzed transfer of \( \gamma \)-phosphoryls from ATP to generate \( [\beta-'^{18} \text{O}] \text{ADP} \) and \( [\beta-'^{18} \text{O}] \text{ATP} \), the number of \( ^{18} \text{O} \) atoms in \( [\gamma-'^{18} \text{O}] \text{ATP} \) is not relevant because in this case \( ^{18} \text{O} \) present in the \( \gamma \)-phosphoryl serves only as a label of the phosphoryl that becomes transferred. Fig. 1 shows the rate at which \( \gamma \)-ATP becomes labeled with 1, 2, or 3 atoms of \( ^{18} \text{O} \) in nonstimulated muscle and in muscle stimulated to contract at 1, 2, or 4 Hz. In resting muscle the \( t_{1/2} \) for \( \gamma \)-ATP to achieve saturation labeling with \( ^{18} \text{O} \) is 2.5 min. This value decreases progressively with the greater rate of ATP utilization occurring with increasing contractile frequency to 1.1 min at 4 Hz. This information is also essential for constructing a metabolic model to provide meaningful estimates of the rates of \( \beta \)-phosphoryl transfer. Another aspect of these results important for developing a relevant kinetic model is that over 97% of the total cellular \( \gamma \)-ATP undergoes labeling with \( ^{18} \text{O} \) when isotopic equilibrium is achieved regardless of the functional state of the muscle. This indicates that virtually all of the ATP in the muscle cell is metabolically active, with respect to its \( \gamma \)-phosphoryl, and has the potential to participate in the \( \beta \)-phosphoryl transfer process in resting or contracting muscle.

Rates of \([\beta-'^{18} \text{O}] \text{ADP} \) and \([\beta-'^{18} \text{O}] \text{ATP} \) Appearance—Experiments were conducted to determine the kinetics of the appearance of \( ^{18} \text{O} \)-labeled \( \beta \)-phosphoryls in ADP and ATP in muscle at rest and when stimulated to contract at frequencies up to 4 Hz (Fig. 2). In non-contracting muscle the rate of appearance of \( ^{18} \text{O} \)-labeled \( \beta \)-phosphoryls in ADP is nearly linear for approximately 20 min when the fraction of the total cellular ADP that becomes labeled nears 30% (Fig. 2A). Very little additional labeling occurs during the subsequent 5-min period of incubation indicating that only about 30% of the total cellular ADP actively participates in \( \beta \)-phosphoryl transfer in resting muscle. In contracting muscle both the rate of \( \beta \)-phosphoryl transfer and the size of the metabolically active pool of ADP increase markedly (Fig. 2A). The rates of appearance of \( [\beta-'^{18} \text{O}] \text{ADP} \) increase with greater frequencies of contraction and asymptotically approach levels of saturation that also increase in magnitude to an apparent maximum representing 65% of the total cellular ADP at 2 and 4 Hz. This plateau, when equilibrium labeling of \( \beta \)-ADP is achieved, is representative of the fraction of the total cellular ADP metabolically active with respect to \( \beta \)-phosphoryl transfer and does not exceed 65% of the total ADP in this muscle under the conditions tested. The increased rates of achieving equilibrium \( ^{18} \text{O} \) labeling of \( \beta \)-ADP with contraction (about 8 min at 2–4 Hz) relative to the rate in resting muscle (20–25 min) cannot be explained by an increase in the \( ^{18} \text{O} \) enrichment of
the phosphoryl donor γ-ATP. The magnitude of the increase in γ-ATP labeling with contraction is small relative to that of β-ADP labeling and the increased labeling of β-phosphoryls with contraction continues at a near linear rate even after the γ-phosphoryl of ATP is almost saturated with 18O (compare Figs. 1 and 2).

The kinetics of appearance of 18O-labeled β-ATP have some of the same characteristics as those of β-ADP, but an additional feature reveals the occurrence of a secondary process. In resting muscle the rate of appearance of 18O-labeled phosphoryls in β-ATP, like that in β-ADP, increases with increasing frequency of contraction. However, in contrast to the β-ADP-labeling kinetics, 18O labeling of β-ATP occurs at two distinct rates. The labeling process extends over a much longer period of time and a much higher percentage, possibly involving the entire cellular content of ATP, and appears to participate in the β-phosphoryl transfer process. The two different rates of β-ATP labeling are characterized by an initial rapid rate occurring during the first 8 min that increases in relation to the frequency of contraction, and a secondary slower rate that is independent of contractile frequency and no different in resting or contracting muscle (Fig. 2B). This slower, apparently fixed rate of β-ATP labeling with 18O is undoubtedly representative of a process whereby [β-18O]ATP exchanges with ATP residing in a cellular compartment(s) in which β-phosphoryl transfer does not occur. This apparent exchange of ATP between compartments of inactive and active β-phosphoryl transfer becomes the rate-limiting determinant of β-ATP 18O labeling after about 8 min. It is noteworthy that the ATP in the apparently inactive β-phosphoryl transfer compartment undergoes relatively rapid metabolism with respect to its γ-phosphoryl (i.e. isotopic equilibrium by 8 min; Fig. 1). For example, in resting muscle 75% of the inactive β-ATP pool is represented by rapidly metabolized γ-ATP. This clearly indicates that β-phosphoryl transfer occurs in a discrete compartment(s) of the cell distinguishable from those in which γ-ATP undergoes only hydrolytic cleavage followed by direct reesterification. Also, since the fraction of the total cellular ATP labeled in the β-phosphoryl increased with increasing frequency of contraction, before this fixed rate of ATP exchange became manifest, it is apparent that in the rapidly contracting muscle the size of the metabolically active β-ATP compartment increased by a magnitude of at least 4-fold (Fig. 2B).

In the same tissues that these measurements of the appearance of 18O-labeled β-ADP and β-ATP were made the concentrations of adenine nucleotides were also determined (Table 1). The results show that the concentrations of all the adenine nucleotides in the contracting muscle were maintained at very nearly the same level as in resting muscle. The constancy of the levels of high energy phosphates under these circumstances indicates that marked increases in adenylate kinase activity occur when the demand for high energy phosphate is increased and when high energy phosphate levels continue to be maintained at their maximum. These observations, the first to monitor rates of adenylate kinase activity in an intact cell, contradict the convention of viewing adenylate kinase as an auxiliary metabolic pathway for salvaging 1 molecule of ATP from 2 spent molecules (i.e. ADP) when the rates of oxidative and glycolytic metabolism are compromised or inadequate.

Estimation of Adenylate Kinase Activity with a Kinetic Model—Since the rate of γ-ATP labeling with 18O is a major determinant and complicating factor for estimating rates of appearance of 18O in the β-phosphoryls of ADP and ATP, an experiment was conducted to minimize this feature by preequilibrating unstimulated tissues with [18O]water. Since the rate of 18O incorporation into γ-ATP in resting muscle proceeds so much more rapidly than the appearance of 18O-labeled phosphoryls in β-ADP and β-ATP, incubation with [18O]water for 4 min resulted in 80% equilibration of γ-ATP with 18O, while only 3.1% of the β-ADP and 6.1% of the β-ATP became labeled with 18O. When muscle was stimulated to contract at 1, 2, or 4 Hz after this preequilibration period the rate of appearance of 18O-labeled β-ADP and β-ATP increased strikingly (Fig. 3) at a rate apparently proportional to the frequency of the stimulus. In the experiment shown in Fig. 3, the maximum possible percentage of β-phosphoryl species labeled with from one to three atoms of 18O was calculated from the [18O]water enrichment and the metabolic compartment sizes to be 53% in ATP and 35% in ADP. Since the data obtained at 2 and 4 Hz begin to approach these limits and were not adjusted for this saturation effect they represent an underestimate of β-phosphoryl transfer rates. Furthermore, the remaining 20% of the γ-ATP, which had not undergone 18O labeling during the preincubation, continued to incorporate 18O during the subsequent incubation and therefore must also be taken into account for a more precise analysis of this kinetic data.

From the results described above the kinetics of 18O incorporation into adenine nucleotide β-phosphoryls are obviously complicated by a number of determinants. They include the rate of 18O incorporation into γ-ATP, the velocity of adenylate kinase-catalyzed phosphoryl transfer, the changing size of the metabolically effective pools of ADP and ATP, and the likelihood of exchange of nucleotides among subcellular compartments. Because of this circumstance, the transformation of the results obtained in Fig. 3 into quantitative information regarding the kinetics of adenylate kinase-catalyzed β-phosphoryl transfer within the cell required computer modeling incorporating these determinants. The model, described in detail under “Experimental Procedures,” allows different velocities to be chosen for the adenylate kinase-catalyzed generation of AMP and its subsequent phosphorylation to produce [β-18O]ADP. The rapid conversion of ADP to generate [β-18O]ATP and hydrolysis of the latter to regenerate [β-18O]ADP are also taken into account. The rates of these reactions are balanced such that the steady state levels of AMP, ADP, ATP are maintained at all times. The model is then used to generate a theoretical time course of 18O appearance in β-ADP and β-ATP for a given adenylate kinase velocity while adhering to the constraints imposed by the time course 18O incorporation into γ-ATP and the limited pool size of metabolic adenine nucleotide.

A series of curves representing the rates of 18O appearance in β-ADP and β-ATP generated by the model are presented in Fig. 4. These curves were generated by varying the adenylate kinase velocity from 1 to 100 nmol·mg protein⁻¹ min⁻¹ while holding the other determinants of β-phosphoryl metabolism constant. In this simulation, not designed to specifically reproduce experimental results, the Fₚ₀₇₀ for β-ADP was 0.65 while that for β-ATP was 0.95 and the ATPase rate was 85 nmol·mg protein⁻¹ min⁻¹. Since the modeling is intended to estimate the adenylate kinase velocity, only the rates for the first 8 min of the simulation are shown; beyond this time the metabolic fraction of ADP and ATP are the predominate determinants of the kinetics rather than the enzyme velocity. From Fig. 4 it can be seen that the general model reproduces virtually all the features of 18O appearance including the lag due to the changing γ-ATP labeling and the steeper slopes and more rapid approach to saturation with increased adenylate kinase velocity. An important outcome of the modeling...
Intact diaphragm muscles were incubated for 4 min at 0 Hz followed by an additional incubation of 4 min (0, 1 Hz), or 2 min (2, 4 Hz) and the sum of the percentages of the total ADP and ATP, as predicted by the modeling. The sum of the total muscle ADP and ATP with [18O]-labeled β-phosphoryls after the 4-min preincubation was 3.65 and 6.08%, respectively, while the ATP hydrolysis was 99.3% of a possible maximum 25%. The total rate of AMP generation refers to AMP generated by adenylate kinase as well as by other pathways that result in [18O]-labeling of the phosphoryl of AMP (0.35 nmol·mg protein·min⁻¹) discussed in the text. The adenylate kinase velocities presented here refer to the amount of AMP that would have to be metabolized specifically by adenylate kinase to form AMP and produced the observed difference in [2-18O] labeling in muscle stimulated at 4 Hz represents the metabolism of more than the entire cellular complement of ATP (30 nmol·mg protein⁻¹) per min. An alternate route of AMP metabolism that must be considered is the deamination of AMP to IMP. This pathway is generally considered to operate in anoxic and severely compromised tissues. We have determined that the rate of IMP generation in muscle stimulated at 4 Hz is approximately 0.76 nmol·mg protein⁻¹·min⁻¹ which represents only about 3% of the total rate of AMP generation at this frequency. Deamination of AMP, therefore, represents only a minor alternative metabolic fate of AMP under these conditions.

### Table I

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In Table I the total rate of AMP generation is equal to the rate of [18O] appearance of the γ-phosphoryls by the rate of appearance of [18O] in the γ-phosphoryls of ATP, the phosphoryl donor. Therefore, as the rate of appearance of [18O]?β-phosphoryls approaches that of the γ-phosphoryls of ATP, the usefulness of the model for predicting adenylate kinase velocity decreases. However, for the physiological functions examined, the model is sufficiently sensitive to obtain good approximations of adenylate kinase velocity. In order to minimize the dependence of the model on the time course of [18O] labeling of γ-[18O]ATP, the results presented in Table I were used to determine the adenylate kinase velocities during the different states of muscle function. It was, therefore, necessary to interpolate only the increase in γ-ATP labeling occurring after the 4-min preincubation. The increase in γ-ATP labeling during the second incubation period was from 4.8 to 9.5% depending on the rate of stimulation.

The results of modeling the data shown in Fig. 3 are presented in Table I. The constraints imposed on the model the experimental results were reproduced quite precisely. In Table I the total rate of AMP generation is equal to the rate of [18O] appearance in β-ADP and β-ATP as predicted by the modeling. This value includes the small (0.35 nmol·mg protein⁻¹·min⁻¹) contribution to AMP formation by non-adenylate kinase pathways (see next section). The adenylate kinase velocity, as presented in the table, refers specifically to the rate at which ADP is metabolized by adenylate kinase to generate AMP and ATP. To obtain this rate 0.35 nmol·mg protein⁻¹·min⁻¹ is subtracted from the overall rate of AMP generation, and the resulting value is doubled since each molecule of AMP generated and phosphorylated by a γ-ATP is preceded by a reaction involving 2 molecules of ADP (i.e. 2ADP → AMP + ATP).

The results demonstrate that there is a nearly direct correlation between increasing frequency of stimulation and adenylate kinase velocity suggesting an association between the ATP hydrolyzed as a result of contraction and accelerated β-phosphoryl labeling. Not yet eliminated is the possibility that even the low rate of adenylate kinase activity observed in non-electrically stimulated muscles is the result of spontaneous fasciculations of individual myofibers that may occur.

Another important feature of the results in Table I alluded to earlier is the near constancy of adenine nucleotide levels under conditions in which accelerated adenylate kinase activity is observed to occur. The apparent decrease in ATP concentration of 3.4 nmol·mg protein⁻¹ at 4 Hz must be viewed in the context of the approximately 600 nmol·mg protein⁻¹ of ATP that were synthesized and utilized during the time in which this small change was measured. Also, the metabolic flux through adenylate kinase in muscle stimulated to contract at 4 Hz represents the metabolism of more than the entire cellular complement of ATP (30 nmol·mg protein⁻¹) per min.
establish precisely the magnitude of the contribution of these reactions to non-adenylate kinase pathways of AMP formation, the rate of $^{18}$O appearance in the $\alpha$-phosphoryl of adenine nucleotides was monitored. By this analysis the non-adenylate kinase rate of AMP formation has been determined to be 0.35 nmol·mg protein$^{-1}$·min$^{-1}$ in non-contacting muscle. More importantly, this rate of $^{18}$O appearance in the $\alpha$-phosphoryl of adenine nucleotides was not affected in diaphragms stimulated to contract. AMP formation by these non-adenylate kinase reactions, therefore, contributes only 5.8, 2.2, and 1.5% to the overall rate of $\beta$-phosphoryl transfer in muscle stimulated at 1, 2, and 4 Hz respectively.

From all the considerations governing $^{18}$O appearance in adenine nucleotides (i.e. observed rates of [\(\beta-^{18}O\)]ADP, [\(\beta-^{18}O\)]ATP, and [\(\gamma-^{18}O\)]ATP appearance, computer modeling, correction for non-adenylate kinase-derived AMP) as well as the requirement that two molecules of ADP are processed for each molecule of AMP arising from adenylate kinase catalysis, the estimates of the total flux of ADP through the adenylate kinase pathway in well oxygenated rat diaphragm are 2.1, 11.3, 23.3, and 47.3 nmol·mg protein$^{-1}$·min$^{-1}$ at 0, 1, 2, and 4 Hz respectively.

Relationship of $\beta$-Phosphoryl Transfer to Oxidative Metabolism—These results indicate that in the intact myofibril the rate of adenylate kinase-catalyzed metabolism of adenine nucleotides is closely related to contractile frequency and/or the increased rate of high energy phosphate utilization associated with stimulated muscle function. They do not suggest a relation between increased adenylate kinase activity and the salvaging of a portion of high energy phosphate metabo-
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In this state of oxygen depletion the rate of ATP consumption was not compromised. ATP utilization rates measured by the rates of appearance of 16O in orthophosphate (i.e., due primarily to hydrolytic cleavage of ATP) and in the phosphor- yls of high energy phosphate intermediates (i.e., reesterification with 16O orthophosphate) showed the overall rate of ATP utilization of resting muscle in nitrogen and oxygen-equilibrated medium to be 133 and 113 nmol·mg protein−1·min−1 and in contracting muscle (1 Hz) to be 214 and 160 nmol·mg protein−1·min−1 in nitrogen and oxygen, respectively. (Details of high energy phosphate utilization rates in rat diaphragm will be the subject of a separate report.)

Since the metabolic pathways for high energy phosphate generation are represented primarily by oxidative phosphorylation and secondarily by glycolysis, an increased rate of glycolysis (11-fold as determined by lactate production) is apparently almost completely compensated for by the reduced oxidative capability in order to maintain nearly the same rates of ATP consumption. The decline in creatine phosphate concentration during the preincubation in nitrogen-equilibrated medium to one-third of the level in fully oxygenated diaphragms and the maintenance of this reduced steady state level throughout the ensuing 8 min indicates that ATP levels and its cellular utilization rate could not have been maintained at the expense of a continually declining mass of creatine phosphate. The greatly increased rate of adenylate kinase activity in the anaerobic state was accompanied by essentially unchanged cellular concentrations of AMP and ADP (not shown).

Since cellular ATP levels and utilization rates were maintained in diaphragms incubated in the nitrogen-equilibrated medium this greatly enhanced rate of β-phosphoryl metabolism catalyzed by adenylate kinase was not involved in salvaging ATP at the expense of ADP and the accumulation of
AMP. If not for the purpose of salvaging, then there must be another importance for the markedly enhanced rates of processing adenine nucleotides through adenylate kinase. The potential importance of adenylate kinase-catalyzed \( \beta \)-phosphoryl metabolism is underscored by the very large proportion of the total cellular \( \gamma \)-ATP hydrolyzed and subsequently processed by this pathway in resting muscle in the oxygen-compromised state. Our calculations show that this amounts to about 80% of the ATP involved in hydrolytic reactions. It must be emphasized that adenylate kinase-catalyzed formation of ATP and AMP from 2 molecules of ADP does not in itself generate \(^{18}O\)-containing phosphoryls. The appearance of \(^{18}O\)-labeled \( \beta \)-ADP (and \( \beta \)-ATP) must, therefore, be interpreted to signify that the reverse of this reaction is tightly coupled to the formation of AMP in a productive way since large fluxes through this pathway occur without substantial changes in cellular adenine nucleotide concentrations and with uncompromised rates of ATP utilization. Rather than behaving as an ATP salvage pathway, adenylate kinase exhibits accelerated activity in association with increased rates of ATP utilization (contraction) and/or when generation of ATP by oxidative metabolism is compromised and compensated for by enhanced glycolytic metabolism.

Availability of Metabolic ADP—The abundant catalytic activity of adenylate kinase in skeletal muscle is currently understood to be regulated by the availability of metabolically effective substrates. It is possible, by using information regarding the kinetic parameters of adenylate kinase-catalyzed \( \beta \)-phosphoryl transfer, to estimate the metabolically effective concentration of ADP that supports the enzyme velocity observed in the present studies to occur in intact skeletal muscle. The kinetic constants for rat diaphragm adenylate kinase activity were determined in unfractionated tissue homogenates assayed with ADP as substrate in the direction of AMP and ATP formation in reaction medium simulating intracellular ionic conditions. The adenylate kinase activity measured under these conditions exhibited a \( K_{m, \text{app}} \) of 243 \( \mu \)M for ADP and a \( V_{\max} \) of 1714 nmol mg protein\(^{-1}\) min\(^{-1}\) (formation of ATP) which represents a catalytic capability approximately 1000 times greater than the velocity observed in the intact muscle cell (i.e. 1.4 nmol mg protein \(^{-1}\) min\(^{-1}\)). If the kinetic parameters exhibited in vitro are representative of those in the intact tissue, the concentration of metabolically effective ADP in the cellular compartment that would be predicted to support the rate of \( \beta \)-phosphoryl transfer ascribable to adenylate kinase in resting muscle is only 0.3 \( \mu \)M. These results indicate that in resting muscle the metabolically effective concentration of ADP at the active site of adenylate kinase is in the submicromolar range, whereas the total content of ADP in the cell, if uniformly distributed, would be equivalent to 1.1 mM. This is consistent with a very restricted distribution of adenylate kinase to discrete cellular compartments to which cellular ADP has very limited access. This information also predicts that the approximate 20-fold increase in the rate of \( \beta \)-phosphoryl metabolism occurring in association with relatively rapid contractile activity (4 Hz) must result from (a) a commensurate increase in the metabolically effective concentration of ADP at the adenylate kinase active site that would be virtually undetected because a 20-fold increase would represent less than a 1% change in the total cellular content of ADP, and/or (b) an increase in the number of discrete cellular compartments of adenylate kinase that become operational. Evidence for the latter may be indicated by the increasingly larger fraction of the total cellular \( \beta \)-ADP that becomes labeled with \(^{18}O\) (at isotopic equilibrium) when the rate of \( \beta \)-phosphoryl transfer increases in relation to increasing frequency of contraction.

The Metabolic Status of Bound and Unbound ADP—A significant proportion of the ADP in skeletal muscle has been described to be tightly bound to actin, myosin, and other proteins (19). The metabolically active concentration of muscle ADP is, therefore, believed to be much lower than would be predicted from measurements of the total cellular content of ADP (20). Although evidence indicates that the ADP in complex with F-actin is metabolically inactive (21), there are also reports to the contrary (22). The capability to monitor the ADP that participates in cellular metabolism by way of \( \beta \)-phosphoryl transfer and pathways resulting in exchange of \( \alpha \)-phosphoryl oxygens provides a means for determining the metabolic status of the unbound and protein-bound forms of this nucleotide.

Protein-bound and unbound forms of ADP were isolated by the differential extraction procedures described under "Experimental Procedures." Precautions were taken to insure separation of these two forms and to minimize interference from secondary association or dissociation that might occur during the isolation. For example, the possibility of exchange of free and bound nucleotide during extraction with Triton X-100 was assessed by determining the extent of exogenous radiolabeled ADP and ATP binding to the detergent insoluble fraction during the extraction procedure. Acid extraction and HPLC purification of the ADP removed from the myofibril preparation indicated that no more than 2-4% of the bound nucleotide could have resulted from exchange during the extraction procedure. Assessment was also made of the potential for ADP metabolism (via adenylate kinase) to occur in the ethanol-containing buffer during the extraction of unbound ADP; insignificant conversion of ADP to AMP and ATP was detected.

Tissues were incubated for 8 min in \(^{18}\text{O}\)water either at rest or with 1 Hz stimulation and the bound and free ADP obtained by the differential extraction procedures were analyzed for \(^{18}O\) content of the \( \alpha \)- and \( \beta \)-phosphoryls. The data obtained from acid-extracted tissues typified previous experiments showing a nearly 3-fold increase in \(^{18}O\) appearance in \( \beta \)-ADP (Fig. 6) and no change in \( \alpha \)-phosphoryl labeling (Fig. 6).
with detergent insoluble myofibrillar protein extracted with tissues is 10-15 times greater than that in the ADP associated phosphoryl labeling with $^{18}O$ shown in Fig. 7 demonstrates that the rate of $^{18}O$ labeling of $\alpha$-ADP in acid extracts of the tissue is much slower than that of $P$-phosphoryl transfer (compare Figs. 2 and 7) and that the rates of $^{18}O$ labeling of $\alpha$-ADP are not different as mentioned earlier.

The results from Figs. 6 and 7 indicate that the phosphoryls of actin-bound ADP do not actively participate in skeletal muscle metabolism. However, these findings do not eliminate the possibility that this species of bound ADP is involved in reversible conversion to ATP without exchanging with the other metabolic compartments of ADP and, therefore, would not undergo $^{18}O$ labeling of its $\alpha$- or $\beta$-phosphoryl.

**DISCUSSION**

These results demonstrate that the $^{18}O$ labeling of cellular nucleotide phosphoryls occurring upon incubation of cells in $[{^{18}O}]$water-enriched medium provides the unique capability to quantitate rates of adenylate kinase-catalyzed phosphoryl transfer in intact skeletal muscle. This analytical approach has the additional power to provide information about compartmental features of this and related adenine nucleotide metabolism. Applying this analytical strategy has uncovered several characteristics of adenylate kinase-catalyzed $\beta$-phosphoryl metabolism in intact muscle cells that could not have been predicted because of the limitations of previous analytical approaches. The new information lays a foundation for developing an entirely new view of the metabolic importance of adenylate kinase in relation to muscle function that coincides in several respects with the scheme proposed by Bessman and Carpenter (6) whereby adenylate kinase fulfills a function of high energy phosphoryl transfer at sites of ATP utilization.

The classical view of this enzyme is that it functions as a scavenger to salvage a molecule of ATP from 2 molecules of ADP that accumulate under conditions when the rate of oxidative or substrate level phosphorylation does not keep pace with the rate of ATP utilization. This involvement of adenylate kinase predicts that the reaction it catalyzes would be at or near equilibrium in the cell and, therefore, assumes that the rates of the forward and reverse reactions are equivalent, occur in the same cellular compartment, and use a common pool of adenine nucleotide as substrate. In red blood cells, which lack mitochondria, the measured content of AMP, ADP, and ATP are representative of the calculated equilibrium for adenylate kinase determined in vitro (20). However, in tissues such as skeletal muscle with abundant mitochondria, the discrepancy between the measured concentrations of nucleotides and those predicted from the adenylate kinase equilibrium is marked. This suggests a more complex relationship of this enzyme with its substrates than originally envisaged.

A major new insight gained is that adenylate kinase-catalyzed $\beta$-phosphoryl transfer is restricted to apparently discrete metabolic compartments within the muscle cell. The results show that in resting muscle a fixed fraction of less than 30% of the total cellular ADP participates in active adenylate kinase-catalyzed $\beta$-phosphoryl metabolism. However, this metabolic fraction of the total cellular ADP increases progressively with increasing frequency of contraction to a maximum of 65%, which was shown to represent all of the nonprotein-bound ADP. This indicates that active adenylate kinase metabolic compartments, which are small or few in number in resting muscle, can increase in size or in number with muscle contraction.

The discreteness of this $\beta$-phosphoryl metabolic unit is most evident from the metabolic behavior of $\beta$-ATP. After the rapid, frequency-dependent rates of $[\beta^{18}O]ADP$ and $[\beta^{18}O]ATP$ appearance there is a secondary, much slower and frequency-independent rate of $[\beta^{18}O]ATP$ appearance. This...
slower monotonic rate of ATP labeling most likely represents a slow fixed rate of exchange between the rapid β-ATP-metabolizing unit and a cellular compartment in which no ATP arises from [3-18O]ADP phosphorylation or does so very slowly. These observations demonstrate (a) compartmentalization of ATP metabolism in cellular locales exclusive for β-phosphoryl transfer but in which γ-phosphoryl transfer also takes place (i.e. in contracting muscle isotopic equilibrium with the γ-phosphoryl of the entire cellular content of ATP is achieved between 4 and 8 min), in contrast to subcellular locales in which γ-phosphoryl transfer occurs with little or no β-phosphoryl transfer catalyzed by adenylate kinase, and (b) that the compartments in which adenylate kinase-catalyzed β-phosphoryl transfer occurs exhibit the capability of generating ATP and maintaining levels of ATP under the physiological state of stimulated muscle contraction.

Experimentally the size of the metabolic compartment is determined by the fraction of the total cellular ADP or ATP that exhibits an 18O-labeled β-phosphoryl when isotopic equilibrium is achieved. However, this fraction of the cellular adenine nucleotide shown to be metabolic does not appear to be representative of the concentration of ADP directly accessible to the catalytic site of the species of adenylate kinase that determines the rate at which β-phosphoryl metabolism will occur. If, for example, all of the ADP that ultimately serves as substrate for β-phosphoryl transfer (equivalent to 1.1 mM) was accessible to the adenylate kinase active site, a velocity of AMP formation in the range of 1200 nmol.mg protein−1.min−1 would be achieved in situ based on the kinetic parameters determined in the cell free system. However, the steady state rate of AMP formation in the intact muscle (i.e. β-phosphoryl flux) is only 1–1.5 nmol.mg protein−1.min−1 in resting muscle and 24 nmol.mg protein−1.min−1 in muscle contracting at 4 Hz. Since the numerous studies of this enzyme activity have not shown that inhibitors or inactive forms of adenylate kinase exist, the limited enzyme activity observed to occur within the cell most likely results from a highly restricted availability of ADP to the rate-determining species of adenylate kinase within each of the discrete metabolic compartments. Since the rate of adenylate kinase-catalyzed β-phosphoryl metabolism accelerates with muscle contraction and these rates of catalysis appear to require an association, and (6) that a source of high energy phosphate has been introduced into the compartment to phosphorylate the [3-14O]ADP and to maintain near constant levels of the adenine nucleotide reactants with little or no net loss of ATP or accumulation of AMP or ADP in spite of the relatively rapid rate at which ATP undergoes hydrolysis. The reactions catalyzed by adenylate kinase cannot provide for high energy phosphate generation (i.e. as oxidative phosphorylation or glycolysis) nor does this enzyme appear to function as is generally thought, to salvage ATP from accumulating ADP.

Net generation of ATP by the adenylate kinase pathway would be accompanied by AMP generation of equal magnitude and, within a short period of time, accumulation of all the ATP in the form of AMP if the ATP produced continues to be hydrolyzed. This obviously is not the outcome when markedly accelerated adenylate kinase activity was observed to occur in association with stimulated contraction in the intact muscle or even when peak rates occur in muscle incubated in oxygen-deprived medium. Instead of generating or salvaging ATP the utility of the adenylate kinase-catalyzed reactions seems to be in its inherent capability to transfer energy-rich β-phosphoryls from one adenine nucleotide to another. Furthermore, this enzyme-catalyzed β-phosphoryl transfer appears to occur in close association with stimulated muscle contraction when ADP would be expected to be made available by components that promote ATP hydrolysis.

These conclusions are consistent with a metabolic scheme proposed by Beaman and Carpenter (6) in which the isozymes of adenylate kinase function as components in a high energy phosphate transfer system. This model describes how two isozymes of adenylate kinase function to maintain the flow of high energy phosphoryls from a primary high energy-producing system to regenerate ATP from the ADP arising from the action of ATPases. According to this proposed scheme, depicted in Fig. 8 (adapted from Ref. 6), two isozymes of adenylate kinase operate in tandem to first generate from 2 molecules of ADP a molecule of AMP, which by the action of the second adenylate kinase is phosphorylated by ATP yielding 2 molecules of ADP that can be rephosphorylated to ATP by the primary energy source. This idea has fundamental correspondence with the results obtained in these studies.

This scheme is consistent with our observations that the rate of AMP generation increases greatly under conditions of increased ATP utilization associated with stimulated muscle

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![Figure 8](http://www.jbc.org/) Proposed scheme for adenylate kinase-catalyzed high energy phosphoryl transfer. This scheme, adapted from Ref. 6, shows a proposed mechanism by which isozymes of adenylate kinase may serve to transfer high energy phosphoryls generated by glycolytic metabolism to ATP utilizing contractile components in muscle. The adenylate kinase phosphoryl transfer system is suggested to operate in parallel with the postulated phosphocreatine shuttle which is coupled to oxidative metabolism occurring in mitochondria.
contraction but that it occurs without the accumulation of AMP. The major source of the ADP that “drives” the activity of adenylyl kinase could be envisaged to be myosin (or Ca2+) ATPase, since this source of ADP has the potential to provide the rate-limiting concentrations of ADP that our results predict must become available during muscle contraction to accelerate adenylyl kinase velocity. Although regulated by the newly generated ADP deriving from ATP hydrolysis, the continual generation of this ADP must result in the rapid phosphorylation of the newly formed AMP to \( [\gamma^3P] \)ADP during what could be represented by the second stage of adenylyl kinase catalysis. This would explain the relatively large quantity of \( [\beta^3P] \)ADP generated while the ADP concentration remains virtually unchanged. \( [\beta^3P] \)ADP within this metabolic unit can then become phosphorylated by the primary energy source to \( [\beta^3P] \)ATP and relatively rapidly equilibrated to \( [\alpha^3P] \)ADP and \( [\beta^3P] \)ATP would be achieved as observed. The secondary, slower rate of \( \beta \)-ATP labeling with \( ^{31} \)O could then occur upon exchange of \( [\beta^3P] \) ATP from the myosin-associated metabolic unit with ATP in another cytosolic compartment.

A feature of the Besaman metabolic scheme that disagrees with the present results relates to the primary energy source replenishing the high energy phosphoryl. In this skeletal muscle the cellular energy-providing system to which this putative adenylyl kinase-catalyzed \( \beta \)-phosphoryl transfer system appears to be coupled is glycolysis. Our results indicate that high energy phosphoryl production by oxidative metabolism may be transiered primarily in the form of creatine phosphate since the rate of \( ^{31} \)O labeling of the phosphoryl of creatine phosphate and that of \( \gamma \)-ATP correspond very closely to that of ATP and are time-correlated. It is most evident from the experiments conducted with nitrogen-equilibrated medium in which the rate of adenylyl kinase-catalyzed \( \beta \)-phosphoryl metabolism markedly increases coincident with enhanced glycolytic flux (i.e. increased lactate production) while steady state levels of cellular ATP were maintained. Also noted (not shown) was a close correspondence between the appearance of \( [\beta^{31}O] \)phosphoryls of muscle lactate and inhibition of muscle lactate. In addition, when dinitrofluorobenzene was used to selectively inhibit creatine kinase-catalyzed \( \beta \)-phosphoryl transfer and generation of muscle lactate. In addition, when dinitrofluorobenzene was used to selectively inhibit creatine kinase-catalyzed \( \beta \)-phosphoryl transfer and generation of muscle lactate. In addition, when dinitrofluorobenzene was used to selectively inhibit creatine kinase-catalyzed \( \beta \)-phosphoryl transfer and generation of muscle lactate. In addition, when dinitrofluorobenzene was used to selectively inhibit creatine kinase-catalyzed \( \beta \)-phosphoryl transfer and generation of muscle lactate. In addition, when dinitrofluorobenzene was used to selectively inhibit creatine kinase-catalyzed \( \beta \)-phosphoryl transfer and generation of muscle lactate. In addition, when dinitrofluorobenzene was used to selectively inhibit creatine kinase-catalyzed \( \beta \)-phosphoryl transfer and generation of muscle lactate.
Evidence for compartmentalized adenylate kinase catalysis serving a high energy phosphoryl transfer function in rat skeletal muscle.
R J Zeleznikar, R A Heyman, R M Graeff, T F Walseth, S M Dawis, E A Butz and N D Goldberg


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