The signal transduction function for oxidative phosphorylation is at least second order in ADP

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To maintain ATP constant in the cell, mitochondria must sense cellular ATP utilization and transduce this demand to F$_2$F$_{-}$ATPase. In spite of a considerable research effort over the past three decades, no combination of signal(s) and kinetic function has emerged with the power to explain ATP homeostasis in all mammalian cells. We studied this signal transduction problem in intact human muscle using $^{31}$P NMR spectroscopy. We find that the apparent kinetic order of the transduction function of the signal cytosolic ADP concentration ([ADP]) is at least second order and not first order as has been assumed. We show that amplified mitochondrial sensitivity to cytosolic [ADP] harmonizes with *in vitro* kinetics of [ADP] stimulation of respiration and explains ATP homeostasis also in mouse liver and canine heart. This result may well be generalizable to all mammalian cells.

Prior work considered that mitochondria behave as a transducer with approximately first order response characteristics (1–4). This means that the response of mitochondrial oxidative phosphorylation (MOP) to a stimulus would follow an approximately hyperbolic relation according to a Michaelis-Menten mechanism for the signal transduction (2, 3). This was used to analyze the apparent order of the kinetic function corresponding to the value of the Hill coefficient, $n_H$ (11). The parameters Max and Min are the $y$-asymptotes of the function, and $x_{0.5}$ is the $x$ value corresponding to half-maximal $y$ (the inflection point).

For comparison, the kinetics of [ADP] stimulation of MOP were analyzed by nonlinear curve fitting using Fig.P software (Elsevier Biosoft). A modified (sigmoidal) Hill function (10) of the form

$$y = \frac{\text{Max} - \text{Min}}{1 + \left(\frac{x_{0.5}}{x} \right)^{n_H}} + \text{Min}$$

(Eq. 1)

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Fig. 1. Stimulation of mitochondrial ATP synthesis flux, \( J_{\text{MOP}} \) (in mmol ATP liter\(^{-1}\) s\(^{-1}\)), by increases in the average ADP concentration (in mM) in forearm muscle cells during contraction (pooled data, \( n = 6 \)). Variables were calculated from \(^{31}\)P NMR spectroscopic data as described elsewhere (8). Over the experimentally achievable range of energy balance states in the muscle, the covariation of \([\text{ADP}]\) and \([\text{Pi}]\) was equally well predicted by any of three relations: (i) \( y = 0.15 \cdot x - 0.03 \) (solid line, \( r^2 = 0.91 \)); (ii) \( y = 0.51 \cdot (x/0.079)/(1 + (x/0.079)) - 0.10 \) (dashed and dotted line, \( r^2 = 0.93 \)); (iii) \( y = 0.29 \cdot (x/0.048)^2/(1 + (x/0.048)^2) - 0.02 \) (dashed line, \( r^2 = 0.93 \)). Inset, extrapolated covariation of \([\text{ADP}]\) and \( J_{\text{MOP}} \) as predicted by each of the three fitted functions over a (nonphysiological) \([\text{ADP}]\) range of 0–0.225 mM.

RESULTS

Dynamic Range of Energy Balance in Skeletal Muscle—The studied range of energy balance states in forearm muscle included the maximal sustainable steady state of energy balance in all subjects as judged from the physiological responses. Typically a maximum occurred at a twitch frequency of 1.6 Hz; higher twitch frequencies resulted in acidosis (8). The calculated rate of contraction coupled ATP hydrolysis in forearm flexor muscle cells at this state was 0.25 ± 0.01 mmol ATP liter\(^{-1}\) s\(^{-1}\) (mean ± S.E., \( n = 6 \)). The matching ATP synthesis flux was mostly mitochondrial (maximally 0.22 mmol ATP liter\(^{-1}\) s\(^{-1}\); Fig. 1) supplemented by a small glycolytic ATP synthesis flux. The measured extent of steady state changes in average PCR content and \( p_{\text{H}} \) in muscle fibers over this range of energy balance states corresponded to approximately 4–5-fold increases of the calculated average \([\text{ADP}]\) and \([\text{Pi}]\) in muscle fibers during contraction (for \( \text{ADP} \) from 0.018 to 0.084 mM (Fig. 1) and for \( \text{Pi} \) from 3.5 to 21 mM, respectively). The calculated muscle cytosolic free energy of ATP hydrolysis, \( \Delta G_{\text{d}} \), decreased from approximately −64 to −54 kJ/mol ATP over this range of energy balance states. The calculated MOP flux \( J_{\text{MOP}} \) increased approximately 28-fold (from 0.008 to 0.22 mmol ATP liter\(^{-1}\) s\(^{-1}\) (Fig. 1). This flux was kinetically limited by \([\text{ADP}]\) rather than \([\text{Pi}]\) in view of the cytosolic concentration ranges of both substrates and the affinity of the ATP and \( P_i \) transporters in the inner mitochondrial membrane (the adenine nucleotide translocator (ANT) (17) and the phosphate carrier (18), respectively).

Analysis of Kinetics of \([\text{ADP}]\) Stimulation of MOP in Skeletal Muscle—Deduction of the apparent order of the kinetic function for ADP stimulation of MOP from these experimental data requires analysis of the scaled rather than the absolute sensitivity of MOP to cytosolic \([\text{ADP}]\) (11), i.e. the percentage of change in flux, scaled to the maximal flux, in response to a percentage of change in stimulus. This crucial point is illustrated in Fig. 1, which shows that both first and second order functions statistically fit the covariation \(([\text{ADP}])_{\text{MOP}} \) equally well over the experimentally accessible range but extrapolate to widely different flux asymptotes (Fig. 1, inset). Thus, analysis of the scaled MOP sensitivity to \([\text{ADP}]\) required knowledge of the \textit{in vivo} maximal and minimal MOP fluxes in the muscle cells. These could not be robustly determined experimentally because energy balance steady states outside the sampled physiological range of the \((\text{ADP}, J_{\text{MOP}})\) covariation did not exist. One possible approach to estimate the flux asymptotes, curve-fitting of an \textit{ad hoc} kinetic function to the data (3), would bias the analysis. We used an alternative approach to estimate the MOP flux asymptotes in the muscle cells that was not biased toward kinetic mechanism; we analyzed the thermodynamic flow-force relation of MOP in the muscle cells (19, 20).

Equation 1 was fitted without any constraints to the covariation \((\Delta G_{\text{d}}, J_{\text{MOP}})\) (Fig. 2A). The fitted maximal and minimal flux were 0.26 ± 0.06 and −0.03 ± 0.02 mmol ATP liter\(^{-1}\) s\(^{-1}\), respectively (± S.E. from regression; \( r^2 = 0.91 \)) (Fig. 2A, inset). This maximum implied that mitochondria in the muscle were stimulated up to 85% of maximal ATP synthesis flux over the full range of sustainable energy balance states (Fig. 2A). The inflection point of the sigmoidal function, \((\Delta G_{\text{d}})_{0.5} \) was −58 ± 1.6 kJ/mol (± S.E. from regression).

The apparent order, \( n_{\text{H}} \), of the kinetic function for cytosolic \([\text{ADP}]\) stimulation of MOP could now be determined by curve-fitting of Equation 1 to the \((\text{ADP}, J_{\text{MOP}})\) data using these values for the flux asymptotes \( \text{Max} \) and \( \text{Min} \) (Fig. 2B). The fitted estimate for \( n_{\text{H}} \) was 2.11 ± 0.14 (± S.E. from regression; \( r^2 = 0.93 \)). Clearly, this result was not compatible with the predicted value (\( n_{\text{H}} = 1 \)) in the generally accepted formalism of Chance (1–4). The fitted estimate for \([\text{ADP}])_{0.5} \) was 44 ± 1 \( \mu \text{M} \), which was approximately equal to half the full range of steady state cytosolic ADP concentrations in the muscle cells (Figs. 1 and 2B).

Analysis of Kinetics of \([\text{ADP}]\) Stimulation of MOP in Other Systems—To test the generality of this \textit{in vivo} result, we also analyzed the \textit{in vitro} kinetics of \([\text{ADP}]\) stimulation of MOP reported for isolated mitochondria (5, 6, 12, 13) (Fig. 2B, inset). Likewise, stimulation of MOP by \([\text{ADP}]\) reported in these \textit{in vitro} studies required in each case a Hill coefficient significantly greater than 1 to explain the kinetics. The range of \( n_{\text{H}} \) values was 2.1–2.9 (2.6 ± 0.2, mean ± S.E., \( n = 4 \)) and not significantly different from the value we obtained for mitochondria \textit{in situ} in skeletal muscle. This result was surprising because the description of approximately first order control characteristics of extramitochondrial \([\text{ADP}]\) (2, 3) had been formulated based on just these studies (12).

To next test if this apparent kinetic order (i.e. between 2 and 3) for transduction of cytosolic \([\text{ADP}]\) to intramitochondrial \( F_1\)-ATPase explains the covariation of cytosolic \([\text{ADP}]\) and MOP flux also in other mammalian cell types, we analyzed the reported kinetics of \([\text{ADP}]\) stimulation of MOP in intact cardiac muscle (14) and liver (15) cells pooled with skeletal muscle data (Fig. 3). The covariation of \([\text{ADP}]\) and the rate of MOP in the pooled data from all three cell types was adequately explained by a transduction function for cytosolic \([\text{ADP}]\) with \( n_{\text{H}} = 2.2 ± 0.4 \) (± S.E. of regression; \( r^2 = 0.73 \)).

DISCUSSION

The main result and novel finding of this study is that the kinetic function for \([\text{ADP}]\) stimulation of MOP in skeletal muscle is approximately a second order function of the form \( J_{\text{MOP}} = \beta([\text{ADP}])^{n_{\text{H}}} \) where \( n_{\text{H}} = 2 \) and \( n_{\text{H}} = 0 \) as has heretofore been assumed (1–4). This implies that the scaled sensitivity of mitochondria to variations in cytosolic \([\text{ADP}]\) is at least order of magnitude greater than has been assumed. This result impacts the understanding of the biochemistry of mitochondria and its integration in the physiology of mammalian cells.
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Apparent Kinetic Order of [ADP] Transduction: Analysis—
The crucial piece of information in the analysis of the apparent kinetic order of the transduction function of cytosolic [ADP] to the mitochondrial matrix was knowledge of the maximal and minimal sustainable MOP fluxes in the muscle cells. Dense sampling of the full physiological domain of the ([ADP], Jp) relation in itself did not allow for discriminating between first or second order (or higher order, for that matter) of the transduction function (Fig. 1). We obtained estimates of the flux asymptotes from analysis of the thermodynamic flow-force relation of MOP (Fig. 2A). This is a well established and valid description of the relation between the flux through a reaction and the concentration of its substrates and products (19, 20). The flow-force relation predicts that under the condition of constant sum of substrate and product concentrations, the flux (or flow) J through a reaction varies in sigmoidal fashion with the thermodynamic driving force $\Delta G$ between maximal forward and reverse rates (19, 20). It was previously shown that this description applies to MOP in muscle (21).

Of utmost importance to the analysis, this approach is by definition unbiased toward the specific kinetic mechanism of a reaction (20). The only prior knowledge about the nature of the flow-force relation of MOP in muscle that was used in the analysis was that this relation is innately sigmoidal (19, 20). The curve fitting of a four parameter sigmoidal function (Equation 1) to the $\Delta G$ vs. $J$ covariation was performed fully unconstrained. The performance of the curve fitting of this function was enhanced by its symmetrical properties.

The fitted estimate of the minimal flux ($-0.03 \text{ mmol ATP liter}^{-1} \text{s}^{-1}$) predicted that there would be net ATP hydrolysis by the mitochondrial ATPase over a nonphysiological range of [ADP] in muscle (i.e. $0 < [\text{ADP}] < 13 \mu M$) if such conditions were to be achieved experimentally in intact cells. This is not unprecedented. Net ATP hydrolysis has been demonstrated in intact isolated mitochondria (20, 22, 23) and significant ATP hydrolysis flux even at maximal net synthetic flux (22, 23), which is entirely consistent with this relation. The flow-force relation predicted net ATP synthesis by MOP over the entire physiological range of [ADP] and $[P_i]$ in muscle corresponding to a $\Delta G_p$ range of approximately $-64$ to $-54$ kJ/mol. Of course, this was fully expected and consistent with mitochondrial function as the primary source of ATP in the eukaryotic cell. On basis of these considerations, we conclude that the estimates of the flux asymptotes from the analysis of the flow-force relation provided a sound basis for analysis of the apparent kinetic order of cytosolic [ADP] transduction in muscle.

Higher Order of [ADP] Transduction: Implications—The result that the apparent kinetic order of cytosolic [ADP] transduction is at least 1 order of magnitude higher than has heretofore been assumed is dramatic and impacts both the understanding of the biochemistry of mitochondria and integrative physiology of mitochondrial function in the cell. According to the formalism proposed by Koshland et al. (24), a Hill coefficient greater than 1 implies amplified sensitivity of mitochondria to variations in cytosolic [ADP]. Sensitivity amplification of enzymes and entire metabolic pathways may be achieved by any of a number of kinetic mechanisms but not a Michaelis-Menten
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mechanism (24). Therefore a fundamentally different molecular transduction mechanism for cytosolic ADP must now be considered.

There is considerable in vitro experimental evidence for allosteric instead of Michaelis-Menten kinetics of adenine nucleotide translocation (13, 25–27). Allosteroism of the translocator ANT could be the mechanistic basis for ultrasensitivity of mito-

chondria to cytosolic [ADP] (24). First of all, our analysis showed that ultrasensitivity of isolated mitochondria to ADP under physiologically comparable conditions of limiting [ADP] and saturating [P"] was abolished upon bypassing the enzyme-
catalyzed translocation of ADP, ATP, and P". We analyzed the published kinetics of [ADP] stimulation of MOP for intact versus digitonin-treated mitochondria (13) and found Hill coefficients of 2.9 ± 0.61, and 1.2 ± 0.05, respectively (± S.E. from regression). MOP flux in each preparation could be 100 and 33% inhibited by ADT inhibition, respectively (13), suggesting that in the latter case cytosolic ADP flux now had direct access to F1,ATPase in a major fraction of the preparation. This suggests that in the latter case cytosolic ADP now had direct access to F1,ATPase in a major fraction of the preparation.

The apparent kinetic order of ADP transduction, nH, may well be different (but ≥ 2) for different cell types (e.g. skeletal versus cardiac muscle cells (Fig. 3)). The mechanistic basis for these possible differences is that specific conditions that affect ANT function such as membrane potential and phospholipid composition (30) may well differ between mitochondria in different cell types. Therefore, although the mechanistic value of n (i.e. the number of strongly cooperative binding sites on the enzyme (11)) for ANT in mitochondria in different cell types may be the same, the kinetic, apparent n value, nH (i.e. the actual operation of the translocation) could differ. It is nH and not n that is measured in kinetic studies.

The new understanding of mitochondrial detection of varia-
tions in cytosolic [ADP] proposed in the present study inte-
grates mitochondrial biochemistry into the physiology of mammalian cells. Second or greater instead of first order of the kinetic function for cytosolic [ADP] transduction has broad implications in cytosolic [ADP] proposed in the present study inte-
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