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Quantitative mathematical expressions for accurate in vivo assessment of cytosolic [ADP] and ΔG of ATP hydrolysis in the human brain and skeletal muscle

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Abstract

Magnetic Resonance Spectroscopy affords the possibility of assessing in vivo the thermodynamic status of living tissues. The main thermodynamic variables relevant for the knowledge of the health of living tissues are: ΔG of ATP hydrolysis and cytosolic [ADP], the latter as calculated from the apparent equilibrium constant of the creatine kinase reaction. In this study we assessed the stoichiometric equilibrium constant of the creatine kinase reaction by in vitro ^{31}P NMR measurements and computer calculations resulting to be: $\log K_{CK}=8.00\pm0.07$ at T=310 K and ionic strength I=0.25 M. This value refers to the equilibrium:

$$PCr^{2-} + ADP^{3-} + H^{+} = Cr + ATP^{4-}$$

We also assessed by computer calculation the stoichiometric equilibrium constant of ATP hydrolysis obtaining the value: $\log K_{ATP\text{-}hyd}=-12.45$ at T=310 K and ionic strength I=0.25 M, which refers to the equilibrium:

$$ATP^{4-} + H_2O = ADP^{3-} + PO_4^{3-} + 2H^{+}$$

Finally, we formulated novel quantitative mathematical expressions of ΔG of ATP hydrolysis and of the apparent equilibrium constant of the creatine kinase reaction as a function of total [PCr], pH and pMg, all quantities measurable by in vivo ^{31}P MRS. Our novel mathematical expressions allow the in vivo assessment of cytosolic [ADP] and ΔG of ATP hydrolysis in the human brain and skeletal muscle taking into account pH and pMg changes occurring in living tissues both in physiological and pathological conditions.

Keywords: ΔG ATP hydrolysis; Creatine kinase reaction; Magnesium; ^{31}P MRS; In vivo magnetic resonance spectroscopy; Apparent equilibrium constant

1. Introduction

In tissues with high energy demands such as muscle, brain and kidney the adenosinetriphosphatase reaction (EC 3.6.1.3) is coupled to the creatine kinase reaction (EC 2.7.3.2) which acts as an energy buffer promptly replenishing the ATP consumed by cellular work. This coupled system is also known as the Lohmann reaction. All the phosphorylated components of the above biochemical reactions exist in cells as mixed ionic species being in equilibrium with their protonated forms and with the complex forms bound to metal cations present in the cell. Chemical equations are written in terms of specific ionic and elemental species, whereas biochemical equa-

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tions are written in terms of reactants that often consist of species in equilibrium with each other and do not balance elements that are assumed to be fixed, such as hydrogen at constant pH [1]. When the pH[1] and the free concentrations of certain metal ions are specified, the apparent equilibrium constant K' for a biochemical reaction is written in terms of sums of species. Various metal ions may be involved, therefore at specified chemical conditions the value of the apparent equilibrium constant K' will be dependent on the concentration of H^+ and of those metal ions that have been taken into account.

In the case of the hydrolysis of ATP (adenosinetriphosphatase reaction), the chemical equation is written as follow:

$$ATP^{4-} + H_2O = ADP^{3-} + PO_4^{3-} + 2H^+ \quad (1)$$

whose equilibrium constant K, defined as stoichiometric constant,[2] depends on temperature and ionic strength only, being independent on H^+ and metal ions concentrations. The biochemical equation for the same reaction is written as:

$$ATP + H_2O = ADP + Pi \quad (2)$$

where ATP refers to an equilibrium mixture of free and protonated forms and of metal complex forms with the main cations present in the cell (obviously the same is referred to ADP and Pi).

Consequently all main Lewis acids present as free ions in the cell cytosol such as H^+, Mg^{2+}, K^+, Na^+ (not Ca^{2+} as it is almost completely bound to proteins like calmodulin and calbinding D-proteins) influence the apparent equilibrium constants of most biochemical reactions and in particular the reactions involved in cellular bioenergetics.

In all tissues, the Gibbs free energy for cellular synthesis, mechanical work, and active transport is calculated from the adenosinetriphosphatase reaction as follows:

$$\Delta G_{ATP-hyd} = AG'^{\circ}_{ATP-hyd} + RT\ln\left(\frac{[ADP][Pi]}{[ATP]}\right) \quad (3)$$

Phosphorus magnetic resonance spectroscopy (^{31}P MRS) can be used to assess in vivo $\Delta G_{ATP\text{-}hyd}$ hydrolysis knowing the $\Delta G'^{\circ}_{ATP\text{-}hyd}$ and by measuring the total concentration of the phosphorylated metabolites involved. However, $MgATP^{2-}$ is the active species in enzyme binding [2] and the energy producing form in active transport [3,4] and muscular contraction [5,6]. As a consequence, only the Gibbs free energy of hydrolysis of this ionic species of ATP is relevant in describing the intracellular energetic status of a tissue [7]:

$$MgATP^{2-} + H_2O = MgADP^- + H_2PO_4^- \quad (4)$$

$$\Delta G_{MgATP^{2-}} = \Delta G^{\circ}_{MgATP^{2-}} + RT\ln\left(\frac{[MgADP^-][H_2PO_4^-]}{[MgATP^{2-}]}\right) \quad (5)$$

Therefore, to characterize the thermodynamic status of a tissue in vivo one should measure the concentrations of these specific species, which is impracticable, or alternatively, formulate an equation in which $\Delta G_{MgATP^{2-}}$ is a function of measurable quantities which in turn are functions of these species concentrations.

Another problem connected with the in vivo assessment of the thermodynamics of living tissues is the calculation of [ADP] from the creatine kinase equilibrium. In vivo assessment of [ADP] in the human brain and skeletal muscle by ^{31}P MRS is essential to the knowledge of the functionality of mitochondrial respiration, hence of the degree of "health" of the tissue [8,9]. However, [ADP] cannot be directly measured in vivo by ^{31}P MRS in whole body magnets due to its low concentration in living tissues and the relatively low spectral resolution of clinical magnets. As a consequence, [ADP] is usually calculated from the creatine kinase reaction [10–15]. Hence, exact knowledge of the apparent equilibrium constant of the creatine kinase reaction (K'_{CK}) is essential for accurate in vivo quantification of [ADP].

Creatine kinase catalyzes the magnesium-dependent reversible transphosphorylation between phosphocreatine (PCr) and ATP according to the reaction:

$$PCr + ADP = ATP + Cr \quad (6)$$

where PCr, ADP and ATP denote the sum of all the ionic, acidic and metal-complexed forms. To calculate the [ADP] from Eq. (6) it is convenient to refer to the apparent equilibrium constant K'_{CK} which takes into account the sum of all forms. K'_{CK} is very useful operationally, but has the drawback of being dependent on H^+ and on metal ion concentrations. As a consequence, K'_{CK} needs to be defined as a function of the concentration of all the Lewis acids to be taken into account.

To date, the commonly used value of K'_{CK} assumes free magnesium ion concentration of 1 mM to calculate [ADP] [16]. However, in both human brain [17–20] and skeletal muscle, the concentration of free Mg^{2+} is far below 1 mM [21–23], and in the human skeletal muscle [Mg^{2+}] changes during exercise and recovery from exercise [23]. Hence in vivo ^{31}P MRS assessment of [ADP] in the human brain and

[1] Here and elsewhere in this article pH is intended as negative decimal logarithm of hydrogen ion molar concentration: pH=−log[H^+], and in analogy pMg=−log[Mg^{2+}].

[2] All the equilibrium constants reported in this article are expressed as a function of concentrations and not of activities.

skeletal muscle suffers from the bias of not taking into account values of $[Mg^{2+}]$ different from 1 mM [24].

The commonly used value of the K'_{CK}=166 was determined by Lawson and Veech [16], by enzymatic assay methods at T=311 K, pH=7, I=0.25 M and $[Mg^{2+}]$=1 mM and later confirmed by enzymatic assay methods by Teague and Dobson [25]. Both studies used an approach that takes into account only some of the magnesium complexes and acidic forms of ATP, ADP and PCr. However, in in vivo systems K^+ and Na^+ ions participate in equilibria with ATP, ADP, and PCr, their intracellular concentration being sufficiently high. Therefore, a more exhaustive approach in the study of tissue bioenergetics should start with the definition of the chemical model best describing the multi-equilibrium system present in the biochemical system under investigation, on which to base an accurate calculation of the apparent equilibrium constants taken into consideration.

In this study we defined a chemical model which: (i) takes into account more magnesium complexes and acidic forms of ATP and PCr than the Lawson and Veech model [16]; (ii) takes into account the complexes formed by ATP, ADP, Pi and PCr with Na^+ and K^+; (iii) makes use of a set of stability and protonation constants critically revised and/or re-determined by potentiometric titration and NMR measurements [20,26].

The aim of this study was the reassessment of K'_{CK} by in vitro ^{31}P NMR measurements and the formulation of quantitative mathematical expressions of $\Delta G_{MgATP^{2-}}$ and K'_{CK}, based on the above chemical model, as a function of quantities measurable by ^{31}P MRS such as [PCr], pH and pMg to afford accurate in vivo assessment of cytosolic [ADP] and free energy of ATP hydrolysis in the human brain and skeletal muscle at any pH and pMg.

2. Materials and methods

Adenosine diphosphate sodium salt, adenosine triphosphate disodium salt, phosphocreatine disodium salt (creatine free) all with a purity > 99%, hepes buffer, and creatine phosphokinase type I from rabbit muscle 310 units/mg protein were purchased from Sigma Chemical Co. (St. Louis, MO, USA); $MgCl_2$, NaCl, NaOH, HCl, and D_2O (99% purity) were from Merck (Darmstadt, Germany).

2.1. Sample preparation for the calibration curve

Samples were prepared in hepes buffer at pa_H=7 and at a concentration of 0.2 M[3]. In all samples a fixed concentration of total Mg^{2+} of 10 mM was achieved adding a fixed volume of a stock solution of $MgCl_2$ whose concentration was precisely determined by flame atomic absorption spectroscopy at 285.2 nm. Different amounts of PCr and ATP were added to have five samples containing PCr in the range 4–20 mM and ATP in the range 10–40 mM. An appropriate variable amount of NaCl was added to each solution to reach a final constant value of ionic strength of 0.25 M. A 20% D_2O was added to provide the lock signal and volume was adjusted to a final 3 mL by adding the appropriate quantity of H_2O to each sample. About 8 mg of the enzyme creatine kinase was added to each sample to make calibration samples as similar as possible to those for experimental creatine kinase equilibrium constant determination. Total concentrations of Na^+ and Cl^- were precisely calculated considering that both ATP and PCr were Na^+ salts and considering the amount of NaOH, HCl, NaCl added to adjust pa_H and ionic strength. The pa_H value of the buffer solution was measured at the same temperature as the NMR experiments (T=310 K) and the pa_H of each sample was controlled before and after NMR spectroscopy by a Radiometer Model PHM26C pH-meter calibrated by standard buffer solutions at pa_H=7 and pa_H=4 with reference values at T=310 K.

2.2. Sample preparation for creatine kinase equilibrium constant determination

Eight samples were prepared as for the calibration curve, the only difference being that ADP instead of ATP was added to PCr, initial PCr and ADP concentration varied in the range 20–40 mM each and were added in molar ratio 1:1 in all samples, and the concentration of hepes buffer at pa_H=7 was 0.4 M. The pa_H value of each sample was accurately determined before and after spectroscopic measurements at T=310 K. About 8 mg of the enzyme creatine kinase was added to the 3 mL sample volume to start the reaction. Preliminary experiments were performed to establish the time required to reach the equilibrium in these experimental conditions. NMR spectra were recorded before and about 5 min after the addition of the enzyme.

2.3. Sample preparation for pK_a determination of ADP

A solution 8 mM of $Na_{1.5}H_{1.5}$ADP was prepared in $(CH_3)_4NCl$ with a final ionic strength of 250 mM. A 10% of D_2O was added to provide the lock signal. The pa_H was varied from about 5 to about 8 by adding a few μL of NaOH 2 M and it was measured at T=310 K before and after NMR spectra recording. The concentrations of all species present were precisely determined taking into account the volumes of NaOH added.

2.4. pK_a determination of ADP[3]

The value of the first protonation constant of ADP was assessed using the computer program HypNMR2000 as described elsewhere [27]. Briefly, the program minimizes by

[3] Here and hereafter pa_H is intended as the decimal negative logarithm of hydrogen ion activity, defined as $\gamma[H^+]$ where γ is the activity coefficient of the hydrogen ion.

an iterative procedure the sum of square residuals between observed and calculated chemical shifts and gives as output the value of the equilibrium constant and the chemical shifts of each species.

2.5. In vitro NMR measurements

NMR measurements were performed on an AMX-400WB Bruker spectrometer operating at 9.395 T. Each solution contained 10–20% of D_2O to provide the lock signal. 1 M methylendiphosphonate (MDP) was used as an external standard for chemical shift reference using a coaxial capillary. ^{31}P spectra were acquired using the WALTZ-16 sequence for proton decoupling with 161.8 MHz spectral frequency; 9800 Hz (60 ppm) spectral width; 32 K data points; 10 μs (60° flip angle) pulse width; 3 s relaxation delay; 1.67 s acquisition time, 50 scans. Dephosphorylation of PCr was negligible during calibration curve samples acquisition, as no Pi signals were detectable on the ^{31}P NMR spectra during the whole session of measurements. The precision of chemical shift measurements was estimated at 0.002 ppm. FIDs were processed with enhancement multiplication using a line broadening of 3 Hz. Peak areas were calculated using an "automatic" routine. A "manual" routine was also performed and the means of three different determinations were compared with those calculated with the "automatic" routine and differences were within 2–4% depending on the peak profile.

2.6. Computer calculations

HYSS is a general computer program [28] for the calculation of the concentration of all the species present at equilibrium in a solution containing a number of reactants. The input data are the formation constants of the species formed in solution, their stoichiometries and information about the composition of the solution. These latter values can be entered as either the total analytical molar concentration of the reactants or the negative logarithm of the free reactant molar concentration. These values can all be held constant (single data point) or some of them can be varied within a predefined interval (titration simulation). Accordingly the output will consist of either a list of the equilibrium concentration of all the species, for single data points, or distribution diagrams and tables of the equilibrium concentration as a function of the variable parameters.

2.7. Calculation of the stoichiometric constant of the adenosinetriphosphatase reaction

The stoichiometric equilibrium constant of ATP hydrolysis ($K_{ATP\text{-}hyd}$) was obtained by HYSS with a trial and error procedure in such a way to have as a result the value of apparent equilibrium constant of ATP hydrolysis reported by Guynn and Veech [29]. We started with an arbitrary value of $K_{ATP\text{-}hyd}$ and changing it at the end of each loop, we ran HYSS as many times as needed to obtain the value of $K'_{ATP\text{-}hyd}=1.63\times10^{5}$ [29]. As a final result we obtained a $K_{ATP\text{-}hyd}=3.52\times10^{-13}$. This value is calculated at T=310 K and I=0.25 M.

2.8. Calculation of the coefficients of cubic equations

The calculation of the coefficients in the interpolation equations derived in this work was performed using the following procedure. The experimental data consisted of the values of the stoichiometric equilibrium constants for the reaction $ADP^{3-}+PCr^{2-}+H^{+} = ATP^{4-}+Cr$ and $ATP^{4-}+H_2O = ADP^{3-}+PO_4^{3-}+2H^{+}$ and the cumulative formation constants of the species reported in Table 1. The concentrations of all the free and complex species present in solution were calculated, using the program HYSS, over a range of values of pH and pMg within the known physiological limits. [PCr] was also used for the calculation of $\Delta G_{MgATP^{2-}}$. Values were calculated for $\mathrm{Log}K'_{CK}$, $\Delta G^{o\prime}_{ATP\text{-}hyd}$ and $\Delta G_{MgATP^{2-}}$ corresponding to each set of pH, pMg and [PCr] values. It was found that these values could be fitted by least-squares to a cubic polynomial. The number of significant figures given for the coefficients are such that if the last digit is

Table 1
Cumulative formation constants (logβ) at T=310 K and I=0.25 M

Species	logβ	Ref.
$HATP^{3-}$	6.79	[20,32]
H_2ATP^{2-}	10.63	[20,32]
$NaATP^{3-}$	1.11	[32]
$KATP^{3-}$	0.98	[32]
$MgATP^{2-}$	4.60	[20,32]
Mg_2ATP	6.21	[18]
$MgHATP^{-}$	8.93	[20,32]
Mg H_2ATP	11.93	[18]
$HADP^{2-}$	6.54	This work
H_2ADP^{-}	10.21	[32]
$NaADP^{2-}$	0.97	[32]
$KADP^{2-}$	0.85	[32]
$MgADP^{-}$	3.22	[32]
MgHADP	7.95	[32]
H PO_4^{2-}	11.69	[20,32]
$H_2PO_4^{-}$	18.43	[20,32]
H_3PO_4	20.64	[20,32]
Na PO_4^{2-}	0.67	[33]
Na HPO_4^{-}	12.29	[33]
K PO_4^{2-}	0.52	[33]
K H PO_4^{-}	12.12	[33]
Mg HPO_4	13.76	[20,32]
$HPCr^{-}$	4.46	[34]
H_2PCr	7.30	[34]
$NaPCr^{-}$	−0.5	[26]
$KPCr^{-}$	−0.3	[26]
MgPCr	1.43	[26]

References are given from which the reported values are corrected for temperature and ionic strength by Van't Hoff and Davies equation respectively.

removed the RMS error increases by 0.001 with $\mathrm{Log}K'_{CK}$ and 0.01 with $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ and $\Delta G_{MgATP^{2-}}$. The quality of the calculated values depends on the quality of the values of the stoichiometric equilibrium constants. These values are known to a precision of about 2 decimal places in the logarithm. The precision of the values of $\mathrm{Log}K'_{CK}$ may therefore be assumed to be similar, that is, 2 decimal places. The precision of $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ and of $\Delta G_{MgATP^{2-}}$ will be of the order of a single decimal place.

3. Results

Fig. 1 reports the scheme of the chemical model used, showing all species involved in the multi-equilibrium system. Four ligands (ATP^{4-}, ADP^{3-}, PCr^{2-} and PO_4^{3-}) and four Lewis acids (Na^+, K^+, Mg^{2+} and H^+) were chosen as basic species.

We considered all known possible equilibria giving rise to a detectable concentration of product species.

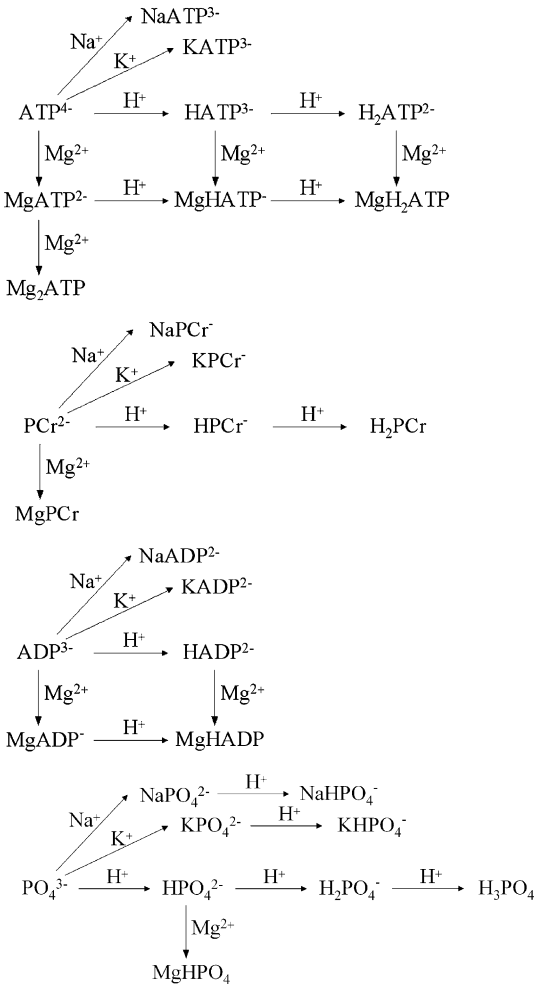

Fig. 1. Scheme of the chemical model representing the main interactions between the four ligands, ATP^{4-}, ADP^{3-}, PCr^{2-}, PO_4^{3-}, and the four Lewis acids, H^+, Mg^{2+}, K^+, Na^+, present in cell cytosol.

The cumulative metal stability constants and cumulative proton association constants of the four ligands (Lewis bases) taken into account in the chemical model are listed in Table 1. The published values of equilibrium constants for the reactions involved in the chemical model chosen were carefully examined and, when needed, the values were corrected for temperature and ionic strength by Van't Hoff and Davies equation respectively. Several of the metal stability and proton association constants of ATP^{4-} and PO_4^{3-} reported (see Table 1) were previously re-determined by potentiometric titration as the results reported by the literature did not exhibit satisfactory agreement [20]. All the metal stability constants of PCr^{2-} were specifically assessed and recently published [26]. The first proton association constant of ADP^{3-} was re-assessed in this work by ^{31}P NMR measurements in $(CH_3)_4NCl$ solution with the aid of the new computer program HYPNMR-2000 [27].

NMR measurements of the creatine kinase reaction performed at equilibrium gave as a result a stoichiometric equilibrium constant $\mathrm{Log}K_{CK}=8.00\pm0.07$ which refers to the equilibrium:

$$PCr^{2-} + ADP^{3-} + H^+ = Cr + ATP^{4-} \quad (7)$$

This is a stoichiometric equilibrium constant (molar concentration quotient) which was obtained at temperature T=310 K and ionic strength I=0.25 M.

This value was obtained as follows: the total concentrations of PCr, and ATP at equilibrium ($[PCr]_{tot}$, $[ATP]_{tot}$) were assessed by NMR measurements. [Cr] was taken equal to $[ATP]_{tot}$, and [ADP] was calculated as the difference between [ADP] initial concentration and $[ATP]_{tot}$. Knowing the value of the stability constants of all the interacting species present in solution, we assessed by HYSS the concentration of all free species at equilibrium. The values in molar concentration of the species PCr^{2-}, Cr, ADP^{3-}, ATP^{4-} for each of the eight solutions tested, and the logarithms of the stoichiometric equilibrium constants and the $[H^+]$ obtained for each solution are reported in Table 2. The $[H^+]$ reported was obtained by experimental pa_H measurements corrected for the activity coefficient calculated by Davies equation for solutions having I=0.25 M for which: $pH=pa_H-0.13$.

Values of K_{CK} obtained for each solution are all in good agreement but solution 1 which, being the most diluted, it is likely to carry a higher bias. Therefore, this measure was rejected and the final value of K_{CK} results from the average of the other seven measurements.

The apparent equilibrium constant of the creatine kinase reaction K'_{CK} was calculated by HYSS at different proton and metal ion concentrations, using the stoichiometric equilibrium constant previously determined. K'_{CK} is defined as:

$$K'_{CK} = [ATP][Cr]/[ADP][PCr]$$

where [ATP], [ADP], [PCr] are the sum of the concentrations of all ATP, ADP and PCr containing species, which, in our

Table 2
Equilibrium concentration (mM) of reactants and stoichiometric equilibrium constant of creatine kinase reaction obtained for each of the 8 solutions used in the ^{31}P NMR experiments

Solution	[ATP^{4-}] (mM)	[Cr] (mM)	[ADP^{3-}] (mM)	[PCr^{2-}] (mM)	[H^+] (M)	Log K_{CK}
1	2.04	17.61	0.66	5.54	3.72×10^{-8}	8.42
2	2.19	17.77	1.46	5.80	3.72×10^{-8}	8.09
3	2.31	17.83	2.20	6.50	3.72×10^{-8}	7.89
4	2.98	19.87	2.43	7.22	3.72×10^{-8}	7.96
5	3.62	21.61	2.77	8.43	3.72×10^{-8}	7.96
6	4.47	23.93	2.91	9.78	3.72×10^{-8}	8.00
7	4.85	26.00	3.10	11.28	3.72×10^{-8}	7.99
8	5.39	28.63	3.12	11.14	3.72×10^{-8}	8.08

experimental conditions, are those reported in the chemical model of Fig. 1.

Program HYSS can work only with stability constants, which means that any equilibrium has to be rearranged having only one product. In the creatine kinase equilibrium this condition was achieved taking Cr as a complex species, as this molecule does not interact with any Lewis acids of the chemical model considered. Therefore the equilibrium (7) has to be written as the formation reaction of creatine as follows:

$$PCr^{2-} + ADP^{3-} + H^+ - ATP^{4-} = Cr \quad (8)$$

and the stoichiometric coefficients to be used in the calculations by HYSS are +1 for PCr^{2-}, ADP^{3-}, and H^+ and −1 for ATP^{4-}.

Fig. 2 reports four different patterns of K'_{CK} plotted as a function of pMg at pH=7 obtained according to the different approach and/or chemical model used. Pattern A was obtained using the Lawson and Veech model [16] which takes into account only the first association constant of ATP, ADP and PCr with Mg^{2+} and H^+. Pattern B was obtained as A using a set of equilibrium constants revised and corrected from those reported in Table 1. Pattern C was the result of our model which in addition takes into account the binding of ATP, ADP and PCr to K^+ and Na^+ and more association constants of ATP and ADP with Mg^{2+} and H^+. Pattern C was obtained at $[Na]_{tot}$=50 mM and $[K]_{tot}$=150 mM. Pattern D shows the behavior of K'_{CK} when in our model the complex Mg_2ATP was neglected.

Fig. 3 reports K'_{CK} as a function of pH plotted at different values of pMg, $[Na]_{tot}$ and $[K]_{tot}$.

To take into account the concomitant influence exerted by pH and pMg on the K'_{CK}, we searched for the simplest equation best fitting a set of 189 data values of K'_{CK} obtained by HYSS in the pH range 5–8 and pMg range 2–4. A cubic equation was found which well describes how the apparent equilibrium constant of creatine kinase changes as a function of pH and pMg at fixed $[Na]_{tot}$ and $[K]_{tot}$:

$$\mathrm{Log}K'_{CK} = a + bx + cy + dx^2 + exy + fy^2 + gx^3 + hx^2y + ixy^2 + jy^3 \quad (9)$$

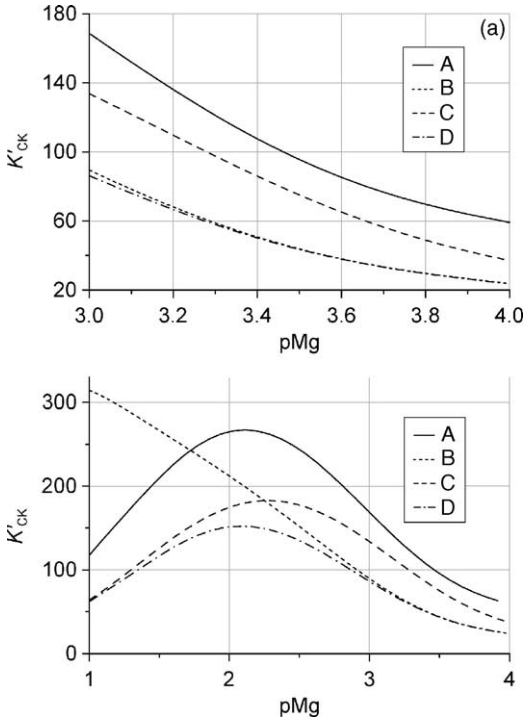

Fig. 2. Apparent equilibrium constant of the creatine kinase reaction (K'_{CK}) plotted as a function of pMg at pH=7. A. Obtained by using the Lawson and Veech model; B. Obtained as panel A using a set of equilibrium constants revised and corrected from those reported in Table 1; C. Obtained by our model at $[Na]_{tot}$=50 mM and $[K]_{tot}$=150 mM. D. Pattern shows the behaviour of K'_{CK} when in our model the complex Mg_2ATP is neglected. (a) Pattern of K'_{CK} in the physiological range of pMg.

where: $x \equiv$pH, $y \equiv$pMg. Table 3 reports the coefficient values obtained at $[Na]_{tot}$=10 mM and 50 mM, and $[K]_{tot}$= 150 mM.

The root mean square errors (RMSE) of the cubic equations were 0.020 and 0.019 for $[Na]_{tot}$ 10 and 50 mM, respectively. In both cases only three data points gave a higher deviation comprised between 0.05 and 0.08 and precisely the values pH–pMg of: 8–2, 7.85–2, and 5–3.75.

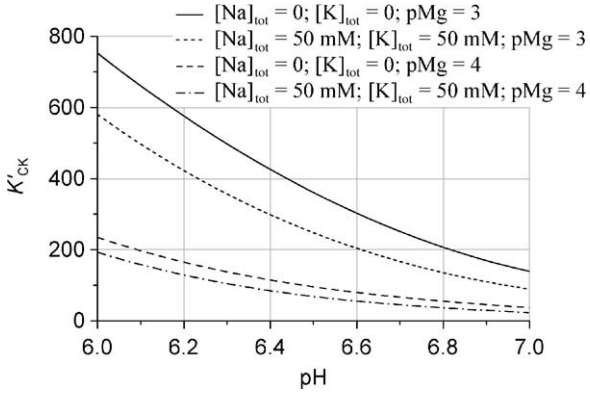

Fig. 3. K'_{CK} as a function of pH plotted at different values of pMg, $[Na]_{tot}$ and $[K]_{tot}$.

Table 3
Coefficient values at $[Na]_{tot}$=10 mM and 50 mM, and $[K]_{tot}$=150 mM of the cubic equation (9) describing the apparent equilibrium constant of creatine kinase in the pH and pMg range 5–8 and 2–4 respectively

$[Na]_{tot}$	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*
10 mM	−0.621	3.2245	−0.9316	−0.5517	0.1347	−0.0341	0.0175	0.03869	−0.1098	0.07188
50 mM	−1.428	3.4744	−0.579	−0.5838	0.0922	−0.0445	0.01925	0.03888	−0.10427	0.07778

Restricting the variation range of pH and pMg to 6–7.2 and 3–3.7 respectively, which is more representative of the pH and pMg physiological fluctuation, and best fitting a set of 36 data values of K'_{CK}, we obtained the coefficient values reported in Table 4, with RMSE of 0.0011 and 0.0010 for $[Na]_{tot}$ 10 and 50 mM respectively, and all data points giving a deviation below 0.003 in both cases.

Fig. 4 reports three-dimensional and curve level plots of the cubic equation obtained in the pH and pMg range 5–8 and 2–4 respectively and at $[Na]_{tot}$=50 mM and $[K]_{tot}$=150 mM.

As it was impossible to determine the equilibrium constant of the reaction of ATP hydrolysis directly, as only traces of ATP would remain at equilibrium, we calculated the apparent equilibrium constant of ATP hydrolysis ($K'_{ATP\text{-}hyd}$) using a different approach. The stoichiometric equilibrium constant of ATP hydrolysis ($K_{ATP\text{-}hyd}$) needed to calculate $K'_{ATP\text{-}hyd}$ as a function of pMg and pH was obtained by HYSS with a trial and error procedure using the experimental value of apparent equilibrium constant reported by Guynn and Veech [29] as described in Materials and methods. As a final result we obtained a $K_{ATP\text{-}hyd}=3.52\times10^{-13}$. This value is calculated at T=310 K and I=0.25 M.

For the same reasons reported for the creatine kinase reaction, the equilibrium:

$$ATP^{4-} + H_2O = ADP^{3-} + PO_4^{3-} + 2H^+ \quad (1)$$

was set up as follows:

$$ATP^{4-} - PO_4^{3-} - 2H^+ = ADP^{3-} \quad (10)$$

and the stoichiometric coefficients to be used in the calculations by HYSS are +1 for ATP^{4-}, −1 for PO_4^{3-}, and −2 for H^+.

By the value of the stoichiometric constant obtained, we calculated $K'_{ATP\text{-}hyd}$ at different proton and metal ion concentrations, and derived the values of $\Delta G'^{\circ}_{ATP\text{-}hyd}$ as a function of pMg and pH.

Figs. 5 and 6 show plots of $\Delta G'^{\circ}_{ATP\text{-}hyd}$ as functions of pMg and pH respectively, obtained at different $[Na]_{tot}$ and $[K]_{tot}$.

Similarly to the creatine kinase equilibrium we obtained a cubic equation fitting 176 data values of $\Delta G'^{\circ}_{ATP\text{-}hyd}$ (kJ mol^{-1}) obtained by HYSS in the pH range 5–8 and pMg range 2–4:

$$\Delta G'^{\circ}_{ATP-hyd} = a + bx + cy + dx^2 + exy + fy^2 + gx^2y + hxy^2 + iy^3 \quad (11)$$

where: $x \equiv$pH, $y \equiv$pMg.

Table 5 reports the coefficient values obtained at $[Na]_{tot}$=10 mM and 50 mM, and $[K]_{tot}$=150 mM.

The RMSE of cubic equations were 0.18 and 0.17 for $[Na]_{tot}$ 10 mM and 50 mM, respectively. The residuals of 166 data points were below 0.3. The introduction of the term x^3 does not decrease the RMSE.

Restricting the variation range of pH and pMg to 6–7.4 and 3–4 respectively, and best fitting a set of 48 data values of $\Delta G'^{\circ}_{ATP\text{-}hyd}$, we obtained a slightly different equation:

$$\Delta G'^{\circ}_{ATP-hyd} = a + bx + cx^2 + dy^2 + ex^3 + fx^2y + gxy^2 + hy^3 \quad (12)$$

with the coefficient values reported in Table 6.

The RMSE of cubic equations were 0.016 and 0.015 for $[Na]_{tot}$ 10 mM and 50 mM, respectively. All residuals were below 0.35. The introduction of the terms y and xy does not decrease significantly the RMSE.

Fig. 7 reports three-dimensional and curve level plots of the cubic equation obtained in the pH and pMg range 5–8 and 2–4 respectively, at $[Na]_{tot}$=50 mM and $[K]_{tot}$=150 mM.

Finally, we derived an expression of $\Delta G_{MgATP^{2-}}$ as a function of [PCr], pH, pMg, all quantities directly measurable by in vivo ^{31}P MRS, to provide a tool to asses the energetic status of intact tissues in vivo. We started from the reaction:

$$MgATP^{2-} + H_2O = MgADP^- + H_2PO_4^- \quad (4)$$

since $MgATP^{2-}$ is the active reactant in enzyme binding and therefore only the free energy of the hydrolysis of

Table 4
Coefficient values obtained at $[Na]_{tot}$=10 mM and 50 mM, and $[K]_{tot}$=150 mM of the cubic equation (9) describing the apparent equilibrium constant of creatine kinase in the pH and pMg range 6–7.2 and 3–3.7 respectively

$[Na]_{tot}$	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*	*j*
10 mM	−9.939	6.264	1.1803	−0.9508	0.0261	−0.5322	0.030367	0.068724	−0.14622	0.1505
50 mM	−11.076	6.5523	1.8084	−0.97275	−0.09749	−0.612	0.031925	0.06872	−0.129047	0.1494

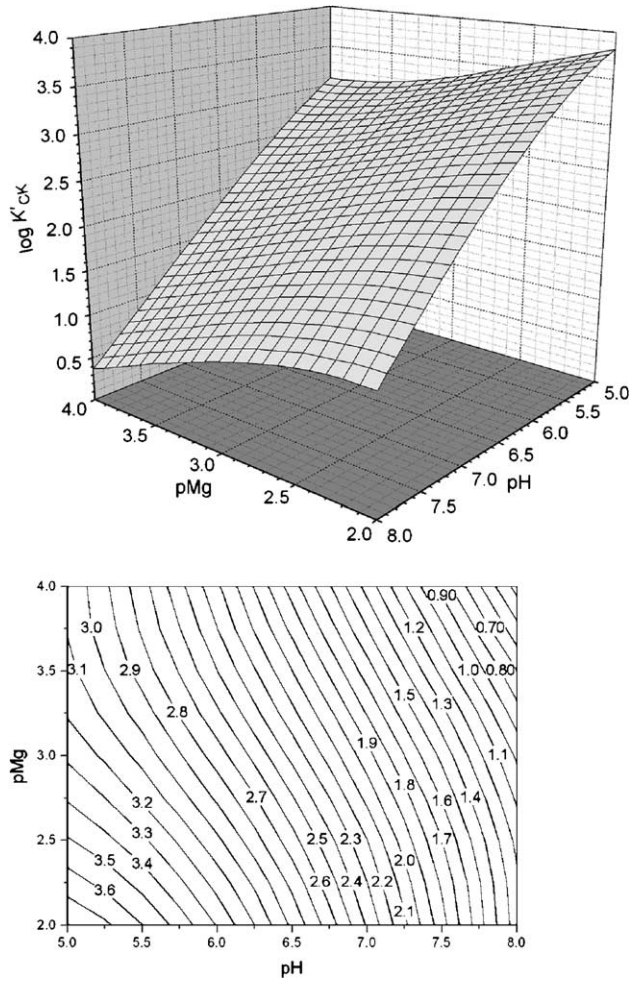

Fig. 4. Three-dimensional and a curve level plots of the cubic equation expressing log K'_{CK} as a function of pH and pMg obtained at $[Na]_{tot}$=50 mM and $[K]_{tot}$=150 mM.

this particular ionic state of ATP is relevant for describing the intracellular energy status of a tissue [7].

Reaction (4) results from the combination of the following reactions:

$$ATP^{4-} + H_2O = ADP^{3-} + PO_4^{3-} + 2H^+ \quad (13)$$

$$\log K = -12.45$$

$$ATP^{4-} + Mg^{2+} = MgATP^{2-} \quad (14)$$

$$\log\beta = 4.60$$

$$ADP^{3-} + Mg^{2+} = MgADP^{-} \quad (15)$$

$$\log\beta = 3.22$$

$$PO_4^{3-} + 2H^+ = H_2PO_4^- \quad (16)$$

$$\log\beta = 18.43$$

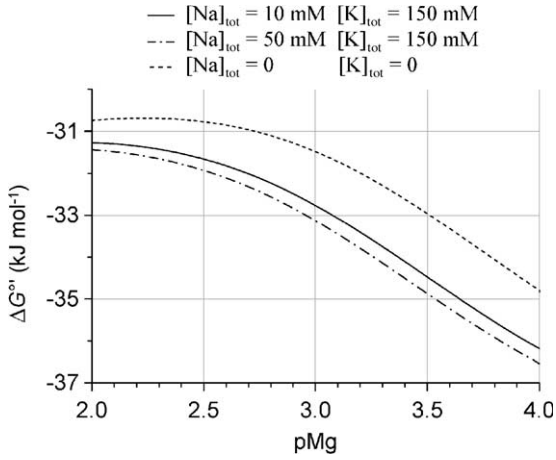

Fig. 5. $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ as a function of pMg, obtained at pH=7 and at different $[Na]_{tot}$ and $[K]_{tot}$.

where (4)=(13)−(14)+(15)+(16). Therefore, the log$K$ of reaction (4) is: −12.45−4.60+3.22+18.43=4.60 and the ΔG° of reaction (4) is −27.31 kJ mol^{-1} at T=310 K and I=0.25 M.

The $\Delta G_{MgATP^{2-}}$ of the hydrolysis of $MgATP^{2-}$ is given by:

$$\Delta G_{MgATP^{2-}} = \Delta G^{\circ}_{MgATP^{2-}} + RT\ln\left(\frac{[MgADP^-][H_2PO_4^-]}{[MgATP^{2-}]}\right) \quad (5)$$

Using the chemical model reported in Fig. 1 and the metal stability and proton association constants reported in Table 1, we obtained the concentration of $MgADP^-$, H_2PO^{4-}, $MgATP^{2-}$ by HYSS at different values of pMg (2.0, 2.5, 3.0, 3.5 and 4.0), pH (5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0) and total PCr mM concentration (36, 32, 26, 20, 12, and 6), which are values within the range of the metabolic fluctuations of the human skeletal muscle, for a total of 210 data points. The calculations were carried out at $[Na]_{tot}$=10 mM and 50 mM and $[K]_{tot}$=150

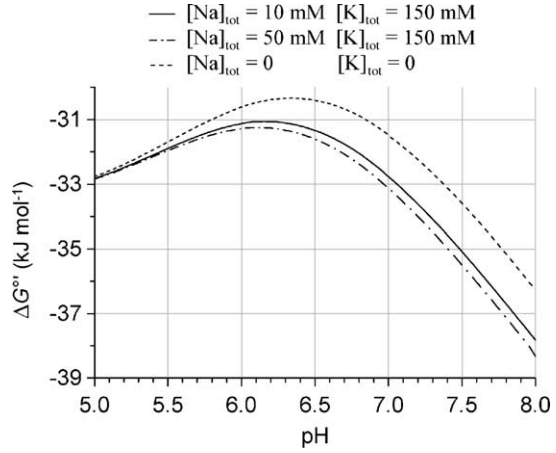

Fig. 6. $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ as a function of pH, obtained at pMg=3 and at different $[Na]_{tot}$ and $[K]_{tot}$.

Table 5
Coefficient values obtained at $[Na]_{tot}$=10 mM and 50 mM, and $[K]_{tot}$=150 mM of the cubic equation (11) describing $\Delta G'^{\circ}_{ATP\text{-}hyd}$ in the pH and pMg range 5–8 and 2–4 respectively

$[Na^+]_{tot}$	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	*i*
10 mM	−74.071	21.3814	−13.723	−2.4018	2.1238	1.3501	0.24662	−0.867	0.44423
50 mM	−75.797	21.454	−11.524	−2.3753	1.8502	0.8782	0.24265	−0.82253	0.47264

Table 6
Coefficient values obtained at $[Na]_{tot}$=10 mM and 50 mM, and $[K]_{tot}$=150 mM of the cubic equation (12) describing $\Delta G'^{\circ}_{ATP\text{-}hyd}$ in the pH and pMg range 6–7.4 and 3–4 respectively

$[Na^+]_{tot}$	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*
10 mM	−195.645	67.143	−8.27476	−1.0172	0.23223	0.44857	−0.9072	0.70664
50 mM	−203.932	71.718	−9.0517	−1.1081	0.28178	0.40478	−0.8273	0.6767

mM. Creatine and inorganic phosphate mM concentrations were set up to satisfy the following conditions: $[Cr+PCr]_{tot}$=42.5 mM, $[ATP]_{tot}$=8 mM according to [30], and $[Pi+PCr]_{tot}$=38.0 mM to take into account the stoichiometric relationship between the variations of PCr and Pi occurring in vivo in the human skeletal muscle in different metabolic conditions [31]. Then, on the basis of Eq. (5), we calculated the values of ΔG (kJ mol^{-1}) for all 210 data points. All the values of $\Delta G_{MgATP^{2-}}$ obtained were fitted by a cubic equation as a function of pH, pMg, and $[PCr]_{tot}$:

$$\Delta G_{MgATP^{2-}} = c_1 + c_2x + c_3y + c_4z + c_5y^2 + c_6z^2 + c_7xy + c_8x^3 + c_9y^3 + c_{10}z^3 + c_{11}x^2z + c_{12}xy^2 \quad (17)$$

where: $x \equiv$pH, $y \equiv$pMg, $z \equiv [PCr]_{tot}$ (mM).

We derived the above 12 parameters cubic equation starting from the general 20 parameters cubic equation (i.e. containing all possible terms) obtained after a minimization procedure that gave an RMSE=0.16 with all residuals below 0.37. Then we dropped the parameters with the highest standard deviation, launched the minimization procedure again and checked whether the RMSE were affected significantly or not. We repeated this loop as many times as to reach the minimum number of parameters that left the value of RMSE not significantly affected.

An analogous 12 parameters cubic equation was obtained for a restricted range of pH and pMg having an RMSE=0.12 with all residuals below 0.22. This equation was obtained performing the calculation on a set of 288 data.

The coefficients of the two equations obtained for two different ranges of pH and pMg and for two values of $[Na]_{tot}$ are reported in Table 7.

Some of the values obtained using the equation for the restricted interval at $[Na]_{tot}$=50 mM are shown as an example in Table 3 where the corresponding values of $[PCr]_{tot}$, pH, pMg used are also reported, together with the $[ADP]_{tot}$ and K'_{CK} calculated using Eq. (9) and $\Delta G'^{\circ}_{ATP\text{-}hyd}$ using Eq. (12) for the restricted interval at $[Na]_{tot}$=50 mM.

We also derived an equation of $\Delta G_{MgATP^{2-}}$ as a function of pH, pMg, and $[PCr]_{tot}$ suitable for the human brain by calculating the concentration of $MgADP^-$, H_2PO^{4-} and $MgATP^{2-}$ by HYSS in the range values: pMg=3.6–4.0; pH 6.8–7.2; and $[PCr]_{tot}$=1–5 mM for a total of 125 data sets.

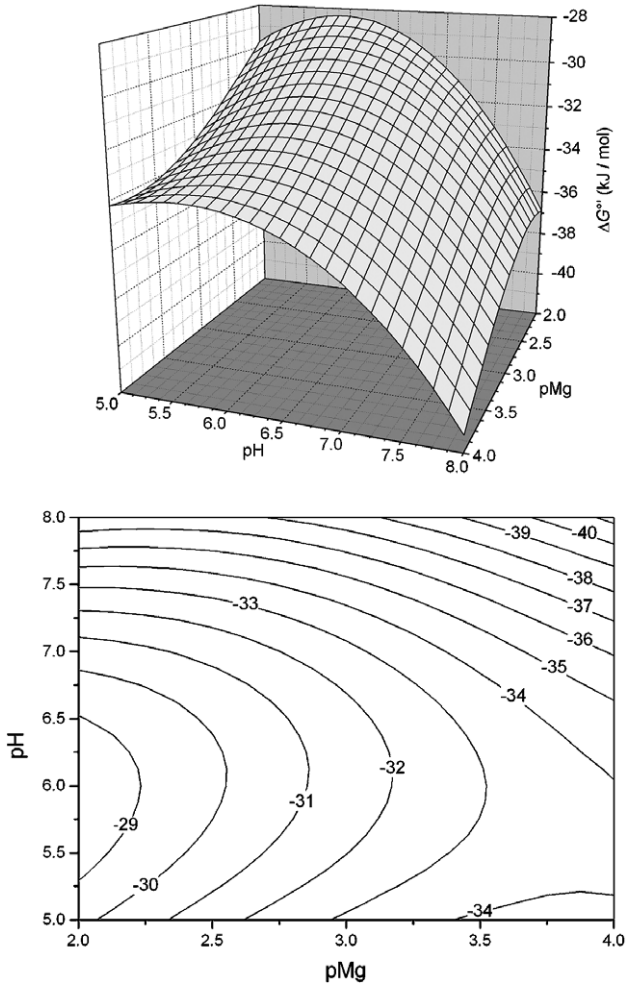

Fig. 7. Three-dimensional and a curve level plots of the cubic equation expressing $\Delta G'^{\circ}_{ATP\text{-}hyd}$ as a function of pH and pMg obtained at $[Na]_{tot}$=50 mM and $[K]_{tot}$=150 mM.

Table 7
Coefficient values obtained at $[Na]_{tot}$=10 mM and 50 mM, and $[K]_{tot}$=150 mM of the cubic equation (17) describing $\Delta G_{MgATP^{2-}}$ in the human skeletal muscle for different pH and pMg ranges

	5 ≤ pH ≤ 8, 2 ≤ pMg ≤ 4		6 ≤ pH ≤ 7.4, 3 ≤ pMg ≤ 4	
	$[Na]_{tot}$=10 mM	$[Na]_{tot}$=50 mM	$[Na]_{tot}$=10 mM	$[Na]_{tot}$=50 mM
c_1	−80.605	−81.042	−98.61	−98.018
c_2	7.423	7.5247	11.026	10.915
c_3	−7.652	−7.215	−3.1837	−2.877
c_4	−1.0014	−1.0012	−1.0005	−1.000
c_5	0.86547	0.83525	0.203	0.188
c_6	0.038867	0.03887	0.03886	0.03886
c_7	1.417	1.3115	0.70635	0.6307
c_8	−0.05439	−0.054377	−0.07296	−0.071952
c_9	0.03573	0.0302	0.03245	0.02827
c_{10}	-0.75623×10^{-3}	-0.7563×10^{-3}	-0.7561×10^{-3}	-0.7562×10^{-3}
c_{11}	-0.0205×10^{-3}	-0.02926×10^{-3}	-0.0364×10^{-3}	-0.05010×10^{-3}
c_{12}	−0.19564	−0.18113	−0.0879	−0.07817

The calculations were carried out at $[Na]_{tot}$=10 mM and 50 mM and $[K]_{tot}$=150 mM. Cr and Pi mM concentrations were set up to satisfy the following conditions: $[Cr+PCr]_{tot}$=11 mM and $[ATP]_{tot}$=3 mM according to [32,33]. The resulting seven parameter equation had an RMSE=0.016 and all residuals below 0.03. Likewise to the equation obtained for the muscle, we started from the general equation containing all 20 terms until reaching an equation with the minimum number of terms and the same RMSE:

$$\Delta G_{MgATP^{2-}} = c_1 + c_2 z + c_3 x^2 + c_4 z^2 + c_5 x^3 + c_6 z^3 + c_7 x^2 y \quad (18)$$

where: $x \equiv$pH, $y \equiv$pMg, $z \equiv [PCr]_{tot}$ (mM).

The coefficients of the equation for two values of $[Na]_{tot}$ are reported in Table 8.

Eqs. (9), (12), (17), (18) and their different variants were all implemented in a Visual Basic program freely available at www.unibo.it/bioclin.

4. Discussion

Our present study provides novel quantitative mathematical equations for in vivo ^{31}P MRS assessment of the apparent equilibrium constant of creatine kinase reaction (K'_{CK}) and ΔG of ATP hydrolysis in the human brain and skeletal muscle, taking into account the pH and pMg changes occurring in living tissues both in physiological and pathological conditions.

First, we defined a chemical model taken as representative of the main interactions between the phosphorylated species involved in the creatine kinase and adenosinetriphosphatase reactions and the main Lewis acids present in the cell cytosol such as H^+, Mg^{2+}, K^+, Na^+ (Fig. 1).

We assessed the stoichiometric equilibrium constant of the creatine kinase reaction, which is function of the free species only, measuring by in vitro ^{31}P NMR experiments the total concentrations of species at equilibrium, and obtaining the equilibrium concentrations of the free species by computer calculations. By this approach we derived the stoichiometric equilibrium constant which in turn allowed the calculation of the apparent equilibrium constant of the creatine kinase reaction (K'_{CK}) at varying pH and pMg. As far as ATP hydrolysis is concerned, we derived the stoichiometric constant from the experimental data given by Guynn and Veech [29] and then calculated $K'_{ATP\text{-}hyd}$ at varying pH and pMg.

All the calculations were performed by HYSS [28] software which, given a set of stability and protonation constants, allows the calculation of concentration of all species at equilibrium. This approach requires a reliable set of stability and protonation constants for any of the equlibria of the chemical model taken into account. Therefore, all the values of stability and protonation constants chosen were critically revised being the result of an accurate selection among (i) those reported in the NIST database [34], (ii) those reported in the literature by other authors [18,35,36], and (iii) those determined by our group by potentiometric titration [20] and NMR measurements [26]. The stability constant values of phosphocreatine with Mg^{2+}, K^+ and Na^+ were specifically assessed for this purpose and recently published [26] as no data were reported in the literature. We also reassessed the protonation constant of ADP^{3-} by NMR at T=310 K. In addition, we investigated whether the

Table 8
Coefficient values obtained at $[Na]_{tot}$=10 mM and 50 mM, and $[K]_{tot}$=150 mM of the cubic equation (18) describing $\Delta G_{MgATP^{2-}}$ in the human brain

$[Na]_{tot}$	c_1	c_2	c_3	c_4	c_5	c_6	c_7
10 mM	−82.8600	−3.5520	1.56370	0.6210	−0.13810	−0.04970	0.710×10^{-3}
50 mM	−81.3849	−3.5528	1.48024	0.6211	−0.13093	−0.04969	0.645×10^{-3}

Table 9
$\Delta G_{MgATP^{2-}}$ values of human skeletal muscle obtained using Eq. (17) at $[Na]_{tot}$=50 mM and the relative values of $[PCr]_{tot}$, pH, pMg

$[PCr]_{tot}$ (mM)	pH	pMg	K'_{CK}	$[ADP]_{tot}$ (μM)	$[ADP]_{tot}$* (μM)	$\Delta G'^{\circ}_{ATP\text{-}hyd}$ (kJ mol^{-1})	$\Delta G_{MgATP^{2-}}$ (kJ mol^{-1})
34	7.0	3.0	89	22	9	−33.1	−63.0
34	7.0	3.5	45	44	15	−34.9	−63.0
34	6.5	3.0	245	8.2	4	−31.6	−64.2
34	6.5	3.5	123	16	7	−33.4	−64.1
6	6.5	3.0	245	200	126	−31.6	−50.1
6	6.5	3.5	123	400	217	−33.4	−50.0
6	7.0	3.0	89	540	288	−33.1	−48.9
6	7.0	3.5	45	1081	504	−34.9	−48.9

K'_{CK} and $[ADP]_{tot}$ were calculated using Eq. (9) and $\Delta G'^{\circ}_{ATP\text{-}hyd}$ using Eq. (12) at $[Na]_{tot}$=50 mM.
$[ADP]_{tot}$* are the values calculated by using the Lawson and Veech's model and reported for comparison.

HEPES buffer used in our experiments displayed any affinity binding to Mg^{2+}, an issue that has not been thoroughly investigated, but we did not obtain any significant value of stability constant.

We compared the pattern of K'_{CK} as a function of pMg obtained by our chemical model (Fig. 2, C) with the one obtained by using the Lawson and Veech model [16] (Fig. 2, A). It is useful to recall that our model differs at three levels: (i) it takes into account more magnesium complexes and acidic forms of ATP and PCr, (ii) it takes into account the complexes formed by ATP, ADP, Pi and PCr with Na^+ and K^+, (iii) it makes use of a different set of stability and protonation constants. Comparison displayed two main differences. First, our model gives systematically lower values of K'_{CK} in the pMg range 2–4, which is physiologically relevant. This result is only partially due to different values of protonation and stability constants used in our calculations, as shown by comparing pattern C with pattern B of Fig. 2, where the latter is obtained by using the Lawson and Veech model with the same values of stability and protonation constants used in our model. This shows that taking into account more complexed species, the resulting values of K'_{CK} are lower in the pMg range 2–4. As pointed out by Kushmerick [37], the importance of K^+ influence (Fig. 3) must be emphasized proving in our calculations to be much stronger than that of Na^+. Second, our model shows a quasi-linear increase at decreasing pMg (i.e. increasing free $[Mg^{2+}]$) instead of reaching a maximum centred at about pMg=2 as in the Lawson and Veech model. This result can be explained by looking at pattern D of Fig. 2, obtained neglecting the complex Mg_2ATP in our model, which also shows a maximum around pMg=2. That such a feature was the result of the influence exerted by Mg_2ATP was one of the hypotheses put forward by Lawson and Veech to explain the discrepancy they obtained between the theoretical curve model and the experimental data at high $[Mg^{2+}]$ [16]. The Lawson and Veech hypothesis is confirmed by our results, which also discard Lawson and Veech's alternative hypothesis that this feature could be due to proton stoichiometry in creatine kinase reaction which is not unity.

The commonly used value of K'_{CK} to calculate [ADP] in living tissues by in vivo ^{31}P MRS (see Ref. [11] for a review) is the one determined by Lawson and Veech at $[Mg^{2+}]$=1 mM [16]. However, the concentration of free Mg^{2+} is far below 1 mM both in human brain [17–20] and skeletal muscle [21–23] where, in addition, it changes during exercise and recovery [23]. The variation of free $[Mg^{2+}]$ in human skeletal muscle during exercise has been reported to be in the range of 0.3– 0.6 mM [23] corresponding to the pMg range of 3.5–3.2, which in turn corresponds to K'_{CK} values from about 40 to 70 at pH 7 according to our present results (Fig. 2a). Moreover, pH changes also affect the K'_{CK}, as shown in Fig. 3, with K'_{CK} increasing at decreasing pH. As a consequence, since a pH decrease during exercise has been shown to be accompanied by an increase in free $[Mg^{2+}]$ [23], at the end of a muscular exercise, K'_{CK} can increase even more than threefold compared to rest. Therefore, for accurate ^{31}P MRS in vivo assessment of cytosolic [ADP] in living tissues, both free $[Mg^{2+}]$ and pH variations must be taken into account, and the appropriate value of K'_{CK} chosen accordingly. It is interesting that increases of K'_{CK} result in decreases of [ADP], thus suggesting that changes in both pH and free $[Mg^{2+}]$ occurring during muscle exercise tend to counteract the [ADP] increase due to [PCr] depletion.

We searched for a mathematical expression which, taking into account the concomitant pH and pMg influences allows a direct assessment of K'_{CK}, in any physiological and pathological condition. We found that a cubic equation best fits the K'_{CK} variations as functions of pH and pMg in the range of 5–8 and 2–4 respectively. The equation obtained by fitting 189 K'_{CK} values has an RMSE sufficiently low to give results with an uncertainty within the experimental error of the stoichiometric equilibrium constant of creatine kinase used in the calculation of K'_{CK}. This means that virtually no extra errors are added in the calculation of K'_{CK} by using the cubic equation, instead of calculating the value directly by HYSS, thus providing a simple tool to obtain K'_{CK} at a given pH and free $[Mg^{2+}]$. In addition by restricting the range of variation of pH and pMg to 6–7.2 and 3–3.7, the key physiological values, we obtained an equation with an even lower RMSE.

We checked the potential influence exerted by Pi increases in the assessment of K'_{CK}, as Pi accumulation occurring in skeletal muscles during exercise represents an

Table 10
$\Delta G_{MgATP^{2-}}$ values obtained using Eqs. (17) and (18) for the restricted interval at $[Na]_{tot}$=50 mM and the relative values of $[PCr]_{tot}$, pH, pMg

Metabolic condition	$[PCr]_{tot}$ (mM)	pH	pMg	$\Delta G_{MgATP^{2-}}$ (kJ mol^{-1})
Resting muscle	34	7.0	3.5	−63.0
Working muscle	6	6.5	3.2	−50.1
Brain	5	7.0	3.8	−62.1

additional binding site for the Lewis acids H^+, Mg^{2+}, K^+, and Na^+ taken into account in our chemical model. Therefore, changes in [Pi] might interfere indirectly with the assessment of the K'_{CK}, although Pi does not take part in the creatine kinase reaction. Interestingly, including Pi in the calculation of K'_{CK} by HYSS and increasing Pi concentration in the range of the physiological values usually reached during muscle exercise, the resulting values of K'_{CK} calculated by HYSS were not significantly affected in the pH and pMg range studied. This is due to the counterplay of the different Lewis acids in the binding of Pi, resulting in a final null effect. This is only the result of a coincidence, which avoided us introducing another variable in the cubic equation of K'_{CK} to take into account Pi changes.

We also studied the variation of the $\Delta G^{\circ\prime}$ of ATP hydrolysis as a function of pH and pMg, and provided an equation analogous to that obtained for the creatine kinase reaction. The influence of pH and pMg was stronger at values above 6 and 3 respectively, with a decrease of $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ as pH and pMg increase. The influence of K^+ was stronger than that of Na^+ both tending to decrease the $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ in the whole range of pH and pMg studied.

In the cell cytosol the creatine kinase reaction is assumed to be at near equilibrium even in exercising muscles if they are in steady state conditions [11]. This hypothesis was for the first time experimentally confirmed by an elegant study performed by ^{31}P NMR magnetization transfer experiments on perfused beating rat heart [38] showing that a requisite for the transfer of energy from mitochondria to ATPase carried out by PCr is the existence of a cytosolic CK functioning at equilibrium. On the other hand, ATP hydrolysis must be far from equilibrium by definition, otherwise no work could be extracted from the reaction to allow the living organisms to survive. Therefore the $\Delta G_{ATP\text{-}hyd}$ is the thermodynamic quantity relevant in assessing the energetic status of a cell in living tissues. ^{31}P MRS can be used to assess in vivo $\Delta G_{ATP\text{-}hyd}$ by measuring the total concentration of the phosphorylated metabolites involved. However, $MgATP^{2-}$ is the active species in enzyme binding [2] and, relevant to our discussion, the energy producing form in active transport [3, 4] and muscular contraction [5,6]. As a consequence, only the Gibbs free energy of hydrolysis of this ionic species of ATP is appropriate in describing the intracellular energetic status of a tissue [7]. In the present work, we derived an expression of $\Delta G_{MgATP^{2-}}$ as a function of $[PCr]_{tot}$, pH, pMg, all quantities directly measurable by in vivo ^{31}P MRS, to provide a tool by which it is possible to characterize in vivo the energy status of intact tissues, also taking into account the possible metabolic fluctuations occurring in skeletal muscle during exercise.

Unlike the equations obtained for K'_{CK} and $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$, which are applicable to any tissue being related to equilibrium constants, the equation for $\Delta G_{MgATP^{2-}}$ must be calculated satisfying the different conditions of $[ATP]_{tot}$, $[PCr+Pi]_{tot}$ and $[PCr+Cr]_{tot}$ which are tissue specific. Therefore we derived two different equations suitable for human brain and skeletal muscle allowing the calculation of $\Delta G_{MgATP^{2-}}$ as a function of $[PCr]_{tot}$, pH, pMg.

All the equations for K'_{CK}, $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ and $\Delta G_{MgATP^{2-}}$ were obtained for concentrations of $[K]_{tot}$=150 mM and $[Na]_{tot}$=10 mM and 50 mM. This choice was made according to the values currently reported in the literature for brain and muscle tissues, which are concordant for $[K]_{tot}$, but variable for $[Na]_{tot}$ which ranges between 10 mM and 50 mM irrespective of the tissue investigated [39–46]. Since the influence of $[Na]_{tot}$ on the values obtained for K'_{CK}, $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ and $\Delta G_{MgATP^{2-}}$ was limited, we decided not to introduce a further variable to take into account possible different values of $[Na]_{tot}$ in the range 10–50 mM. Therefore, to calculate the values of K'_{CK}, $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ and $\Delta G_{MgATP^{2-}}$ for any $[Na]_{tot}$ value within the above range it is reasonable to perform a linear interpolation.

It must be underlined that we obtained these equations following a heuristic procedure, therefore all the coefficient values obtained for each equation do not have any specific physical meaning. These equations just allow the calculation of the values of K'_{CK}, $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ and $\Delta G_{MgATP^{2-}}$ at the specified conditions.

We also found that $[PCr]_{tot}$ is the variable mainly influencing the $\Delta G_{MgATP^{2-}}$ much more than pH and pMg, the latter showing little influence (Table 9). As a consequence, the depletion of $[PCr]_{tot}$ occurring during muscle contraction strongly affects $\Delta G_{MgATP^{2-}}$, which for a depletion of $[PCr]_{tot}$ above 80%, can reach values of −50 kJ/mol independently on the pH and pMg (Tables 9 and 10). This is a surprising result as $\Delta G^{\circ\prime}_{ATP\text{-}hyd}$ was in fact more affected by both pH and pMg. This result reinforces the concept that the cytosolic $\Delta G_{MgATP^{2-}}$ is a tightly controlled variable [7] and becomes even more evident if we compare the $\Delta G_{MgATP^{2-}}$ values calculated for the human resting skeletal muscle and brain by Eqs. (17) and (18), which are −63.0 kJ/mol and −62.1 kJ mol^{-1} respectively (Table 10), thus being very similar although the $[PCr]_{tot}$ is much lower in the brain than in the skeletal muscle. The similarity between the $\Delta G_{MgATP^{2-}}$ values obtained in human skeletal muscle and brain agrees with the calculation made by Roth and Weiner [7], although they obtained slightly different values of $\Delta G_{MgATP^{2-}}$ and assumed a $[Mg^{2+}]$=1 mM both in brain and in skeletal muscle. On the other hand, the change of $\Delta G_{MgATP^{2-}}$ over 20% that we found in the skeletal muscle after heavy exercise largely differs from their calculations which predicted changes around 5% [7].

On the basis of these results and with the aim of this new equation it will be interesting to investigate the relationship between $\Delta G_{MgATP^{2-}}$ and fatigue in future studies.

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