16 Organization of the CK system in the perfused rat heart. I. Magnetization transfer evidence of a mitochondrial ATP compartment

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Introduction

Magnetization transfer techniques (saturation, inversion) have often been applied to the determination of myocardial creatine kinase (CK) fluxes in the perfused heart or *in vivo*. Since the heart is in steady state, both the forward Ff (PCr→ATP) and reverse Fr (ATP→PCr) fluxes should be equal in a two-site model. However this simple model which considers the cell as an homogenous solution has been recognised as an oversimplification of the cardiac CK since both Ff and Fr were often found unequal. The observed flux discrepancy was proposed to result either from the subcellular compartmentation of substrates or enzymes, or from the exchange of ATP with other phosphorus species for example Pi [1-5]. We previously showed in an in vitro CK system, that the full time analysis of an inversion of PCr (inv-PCr) or of ATP(inv-ATP) can independently reveal the presence of an ATP compartment not involved in exchange with PCr through CK. This approach applied to the beating control rat heart is consistent with the existence of an ATP compartment of about 20% which is not directly exchanging through CK [6].

Aim

We postulated that the ATP compartment revealed by NMR might be mitochondrial ATP. Indeed, depending on mitochondrial activity and work a non negligible fraction of metabolites is known to be sequestered in the mitochondria [7]. To test this hypothesis, we used various physiological conditions designed to modify CK metabolite concentrations and their subcellular distribution. Ff and Fr were measured by inversion transfer of PCr or ATP. In parallel the subcellular distribution of CK metabolites was assessed by fractionation in non aqueous medium [7]. The flux discrepancies were related in both inversion protocols to the amount of ATP sequestered in mitochondria. Under saturation of Pi (sat-Pi) which masked the contribution of ATP-Pi exchange, inversion protocols allowed a direct determination of the ATP compartment which was in good agreement with mitochondrial ATP measured biochemically.

Theory

CK fluxes measurement by inversion transfer experiments (two-site model)

Both fluxes $Ff = k_f M^{\infty}_{PCr}$ and $Fr = k_r M^{\infty}_{ATP}$ can be determined by a full time analysis of the return to equilibrium of the inverted and non inverted species [8]. The evolution of PCr and ATP magnetizations (M_{PCr} and $M_{\gamma ATP}$), which depends on the apparent rate constant of the chemical exchange between species (k_f, k_r) and on the intrinsic magnetic relaxation parameters (T_{1PCr}, $T_{1\gamma ATP}$), is described in a two-site exchange system by the solutions of McConnell equations:

$$M_{PCr}(t) = M^{\infty}_{PCr} + C_1 \exp \lambda_1 t + C_2 \exp \lambda_2 t \tag{16.1}$$

and

$$M_{\gamma ATP}(t) = M^{\infty}_{\gamma ATP} + C_3 \exp \lambda_1 t + C_4 \exp \lambda_2 t \tag{16.2}$$

where the different constants depend on k_f, k_r, T_{1PCr} and $T_{1\gamma ATP}$. Thus the complete analysis of the time evolution of the inverted and non inverted species give access to both fluxes in the same experiment: a decisive advantage over saturation transfer protocols.We showed, in vitro, that a classical analysis (two-site) results in incorrect determination of the kinetic parameters in case of the presence of a compartment of ATP which is not exchanging with PCr through CK (f_{ATP}). As the size of f_{ATP} increased the ratio Ff/Fr decreased below unity in an inv-PCr protocol and increased (>1)in case of an inv-ATP allowing to quantify the size of this ATP compartment [6].

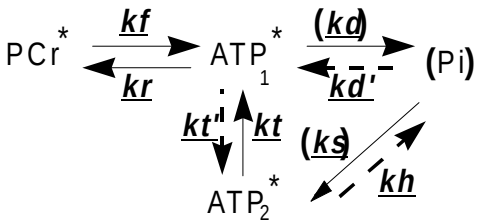


Fig. 16.1 The scheme shows the model used and its simplification under sat-Pi. In parenthesis: compartments and exchanges suppressed by sat-Pi; $\star$ = new steady state of PCr and ATP.

Model accounting for the existence of an ATP compartment and an ATP $\Leftrightarrow$ Pi exchange

Contrary to PCr which is only metabolized by CK, ATP is involved in other myocardial reactions, mainly ATP $\Leftrightarrow$ Pi exchange. Thus in the myocardium both an ATP compartment (ATP_2) and ATP $\Leftrightarrow$ Pi exchange must be considered. The presence of the latter precludes a direct determination of ATP_2, however its influence can be eliminated by a continuous saturation of Pi [4].

Thus, analyzing the inversion protocols under sat-Pi, allows to extract the size of the ATP compartment not exchanging directly through the CK reaction by solving:

$$\frac{d}{dt}\begin{pmatrix} M_{PCr} \\ M_{\gamma ATP1} \\ M_{\gamma ATP2} \end{pmatrix} = \begin{pmatrix} -k_{1f} & k_r & 0 \\ k_f & -k_{1r} & k_t \\ 0 & k'_t & -k_{1t} \end{pmatrix} \begin{pmatrix} M_{PCr} - M^{\infty}_{PCr} \\ M_{\gamma ATP1} - M^{\infty}_{\gamma ATP1} \\ M_{\gamma ATP2} - M^{\infty}_{\gamma ATP2} \end{pmatrix}$$

where

$$\begin{cases} k_{1f} = k_f + \frac{1}{T_{1PCr}} \\ k_{1r} = k_r + k_d + k'_t + \frac{1}{T_{1\gamma ATP_1}} \\ k_{1t} = k_t + k_h + \frac{1}{T_{1\gamma ATP_2}} \end{cases}$$

Materials and methods

Physiology Langendorff perfused rat hearts were studied in isovolumic conditions of work, at constant flow. Hepes buffered (20mM) perfusate contains 10mM acetate as substrate (pH_o = 7.35, T = 36.5°C, O_2 = 100%). In control group contractility, estimated by the rate pressure product (RPP) was 3.98 ± 0.12 10^4.mmHg.beats^{-1}.min^{-1} (mean ± sem, n = 20) corresponding to an ATP synthase activity of 2.3 ± 0.01 mM.s^{-1} as estimated from the oxygen consumption measurement.

Three other experimental groups were designed to change the work performed and/or the distribution of metabolites in the subcellular compartments: group 2DG, cyanide and hypoxia. In all groups ATP and PCr content were reduced by

about 50%. In normoxia the depletion induced by 2-deoxyglucose, 2DG, hardly affected RPP as previously described [9] (at most 10%; $n = 20$). Partial ATP synthesis inhibition (ca 60% of RPP reduction) was achieved either by addition of cyanide ([NaCN] = 0.15 mM; $n = 17$), or by hypoxia (30% of O_2 in perfusate; $n = 7$).

NMR INNOVA Varian 9.4T, acquisition = 4 K, LB = 20 Hz, spectral width = 10000 Hz, 80° pulse. *Inversion transfer*: inversion of either PCr, inv-PCr, or γATP, inv-ATP, (sinc pulse of 15 ms), followed by a variable delay (0 to 10 s) before the sampling pulse and 10 s of relaxation, number of scans 24 (4 scans cycling 6 times through the whole protocol).

Analysis Errors resulting from the use of a simple 2-site exchange model (i.e., neglecting ATP⇔Pi exchange and ATP compartment) were first revealed by adjustment with a double exponential fit. In a second time, simultaneous adjustment of both inv-PCr and inv-ATP protocols was performed under sat-Pi. All analysis were performed using the Levenberg Marquardt method implemented under LabVIEW (Nat. Inst. USA). The adjusting parameters are described in the theory section.

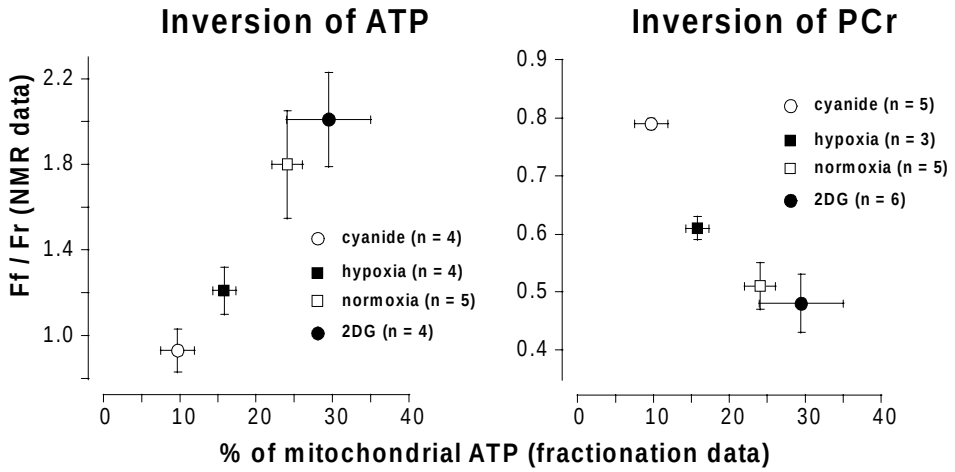
Subcellular distribution of metabolites At the end of the NMR experiment hearts were freeze clamped. Five hearts of each group were freeze dried. The distribution of CK metabolites in cytosol and mitochondria was analyzed by tissue fractionation technique in non aqueous solvent as previously described [7].

Results and discussion

The discrepancy between forward and reverse CK flux is related to mitochondrial ATP

Both inversion of PCr and ATP protocols were performed in the various experimental groups. At first the classical 2 site exchange model was used to estimate flux ratios Ff/Fr. Ff/Fr was <1 in all groups upon inv-PCr (range: 0.48 to 0.79). Ff/Fr was ≥1 (range: 1 to 2) when ATP was inverted. This is indeed the behavior expected from the existence of an ATP compartment, f_{ATP}, not directly exchanging with CK. The relationship between Ff/Fr measured by NMR and the amount of mitochondrial ATP determined by subcellular fractionation in non aqueous medium (in % of total ATP) is shown below for both inversion protocols.

As the mitochondrial ATP compartment increased in the various groups the deviation from equality of forward and reverse flux increased. This firstly confirms that our NMR protocols provide evidence for the existence of an ATP not directly



involved in CK and secondly suggests that this ATP might be located in the mitochondria. Notice however that, in this protocol, the existence of an ATP⇔Pi exchange precludes a direct quantification of f_{ATP}.

The ATP fraction detected by NMR is similar to mitochondrial ATP

Continuous saturation of Pi resonance was previously used in saturation transfer protocols to eliminate the contribution of the ATP⇔Pi exchange [4]. Application of this method to the inversion protocol allowed the correct determination of Ff and f_{ATP} in each experimental group.

Condition	2DG	Control	Hypoxia	Cyanide
NMR determination by inv-PCr under Pi saturation				
– Ff mM/s	6.5 ± 0.9	6.9 ± 0.8	3.4 ± 0.4	5.8 ± 1.2
– f_{ATP}% total	33	24	14	9
Determination by subcellular fractionation				
– ATP mito % total	30 ± 5	24 ± 2	16 ± 2	10 ± 2

(n=4-5)

The Ff measured in inv-PCr under saturation of Pi was similar to those we observed in the same physiological conditions by conventional time dependent saturation transfer of ATP [9,10]. Moreover, excellent agreement was found between the ATP compartment observed by NMR and by subcellular fractionation in the various physiological conditions. Notice that the exchange between $ATP_1 \Leftrightarrow ATP_2$ can not be evidenced (high confidence interval): in terms of NMR ATP_2 behaves as isolated from CK under Pi saturation. To understand the relationship of intracellular ATP compartments, we suggest a simultaneous global analysis of the 4 inversion protocols used here (see part II). This approach might help to obtain

the kinetic parameters of ATPase, ATP synthase and to eventually to separate the contribution of CK isoforms and ATP diffusion in various working conditions.

In conclusion we showed by inversion transfer a discrepancy between the forward and reverse myocardial CK flux which can be accounted by the presence of an ATP compartment isolated from CK. Masking the contribution of ATP⇔Pi exchange allowed the direct determination of this ATP compartment which is compatible with mitochondrial ATP measured biochemically. This experimental approach might improve our understanding of the complexity of energy transfer in myocardium.

References

1. Nunnally R.L. and Hollis D.P. (1979) *Biochemistry* **18**, 3642–3646.
2. Meyer R.A., Kushmerick M.J. and Brown T.R. (1982) *Amer. J. Physiol.* **242**, C1–C11.
3. Koretsky, A.P., Basus V.J., James T.L., Klein M.P. and Weiner M.W. (1985) *Mag. Res. Med.* **2**, 586–594.
4. Ugurbil, K., Petein M.A., Maiden R., Michurski S.P. and From A.H.L. (1986) *Biochemistry* **25**, 100–108.
5. Spencer R. G., Balschi J.A., Leigh J.S. and Ingwall J.S. (1988) *Biophys. J.* **54**, 921–929.
6. Joubert, F., Mateo, P., Gillet, B., Beloeil, J.-C. and Hoerter, J.A. (1999) *Biophys. J.* **76**, A308.
7. Soboll S. and Bunger R. (1981) *Hoppe-Seyler's Z. Physiol. Chemie* **362**, 125–132.
8. Led , J.J. and Gesmar H. (1982) *J. Mag. Res.* **49**, 444–463.
9. Stepanov, V., Mateo, P., Gillet, B., Beloeil, J.-C., Lechene, P. and Hoerter, J.A. (1997) *Am. J. Physiol.* **273**, C1397–C1408.
10. Mateo, P., Stepanov, V., Gillet, B., Beloeil, J.-C. and Hoerter, J.A. (1999) *Am. J. Physiol.* **277**, H308–H317.

17 Organization of the CK system in the perfused rat heart. II. Metabolic fluxes estimated by simultaneous analysis of several magnetization transfer protocols

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Introduction

Despite considerable efforts devoted to the study of creatine kinase there is still some controversies about the physiological interpretation of NMR measured CK flux. The main problem in the interpretation of NMR data in vivo arises from the oversimplification of a highly organized system: the assumption that the cell is an homogenous system containing enzymes and metabolites whose uniform concentration dictate the kinetics of the enzymes. However part I suggests that (i) a protocol of inversion transfer under saturation of Pi was able to directly quantify a compartment of ATP not involved in CK reaction, (ii) mitochondrial ATP measured by fractionation technique is a good candidate for this ATP compartment which in terms of NMR appears isolated from the CK flux. Complexity in the NMR analysis has indeed been suggested to arise from the presence of ATP involved in exchange with Pi, from ATP compartmentation or from CK compartmentation since in the myocardium about 50% of CK is found in close vicinity of ATPases or adenine nucleotide translocator.

We propose that a simultaneous analysis of several protocols of magnetization transfer might allow to extract detailed information on the energy fluxes of the myocardial cell that can not be obtained by one protocol due to the amount of unknown variables.

Methods

Experimental

This approach is shown here for hearts using acetate as substrate (i.e., negligible glycolysis activity) in control conditions (see part I for physiological conditions). Metabolite contents and work were similar in all series: the pooled metabolite concentrations (in mM) were ATP = 6.8 ± 0.3, PCr = 12.3 ± 0.3, Cr = 11.6 ± 0.3, Pi = 2.9 ± 0.2, pH_i = 7.10 ± 0.01 (n = 20). An ATP synthesis rate of 2.3 mM/s was calculated from the oxygen consumption measurement (QO_2 = 10.1 ± 0.3 μmole O_2/min/gWW) assuming a P/O ratio of 6.

Four groups of hearts (n = 5 in each group) were used: in each group one inversion transfer protocol was applied either inversion of γATP (inv-γATP), inversion of PCr (inv-PCr), or inv-γATP, inv-PCr under continuous saturation of Pi (see part I for experimental conditions)

Due to the small size of Pi peak in hearts using acetate, it was impossible to reliably measure the kinetics of ATP ⇔ Pi.

Analysis

The kinetic description of a multi-exponential relaxation process implies the determination of the time constants and amplitudes of each component. In the classical analysis, each NMR protocol must be described by such a set of parameters. However, given a model of exchanging pools of metabolites, the rate constants only depend on the organization of the compartments and must be the same whatever the measured process is. Our suggestion is to use this property to reduce the total number of parameters to be determined by simultaneously fitting several protocols. We developed a program to concatenate the measurements of the four NMR protocols and to fit the response of a model to the concatenated protocols by the method of Levenberg-Marquardt. The algorithm was implemented using LabVIEW (Nat. Inst., USA). The values are given with their confidence intervals and with the X^2 of the fit.

The benefit of the method is a powerful increase in the determination of physiological parameters, the drawback being that the parameters are model-dependent. Therefore several models had to be investigated.

In all models four constraints were imposed: PCr is only exchanging with ATP, total metabolite concentrations were fixed to their experimental values, Pi was in steady state (ATP → Pi = Pi → ATP), T_{1Pi} was imposed to 1s [1].

The criteria for selecting a model were: (i) low confidence interval on variables, (ii) minimal incertitude on the fit (estimated by the X^2), (iii) agreement between results of the computation and experimental data obtained by other methods than NMR (namely ATP synthesis estimated from oxygen consumption and size of the

ATP compartment estimated by subcellular fractionation), (iv) computed relaxation parameters of PCr and γATP in the range found in literature (for PCr from 2.5 to 4 s, and for γATP from 0.5 to 1.2 s), (v) consistency of the model with prior knowledge of the system from literature.

Results and discussion

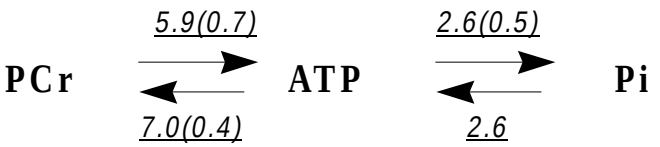
As a first approach we applied 4 simple models of phosphorus moieties exchange implying PCr, ATP and Pi to the analysis of the experimental data. Model I is the classical three-site exchange with an ATP ⇔ Pi exchange, model II considers the existence of an ATP compartment isolated from CK turnover, model III explores the relation of this compartment with CK, model IV considers the existence of specific intracellular localization of CK isoforms.

Analysis of the experimental data of the inversion protocols

Model I: three-site exchange

Since ATP, as opposed to PCr, is involved in other cellular exchanges a two-site exchange is insufficient to analyze myocardial CK which has been suggested to be better described by a three-site exchange model [2]. In control myocardium we found no evidence of myokinase activity and glycolysis is negligible due to the utilization of acetate as substrate. The most relevant flux is the exchange of ATP with Pi through ATPases and ATP synthesis due to sustained mechanical activity.

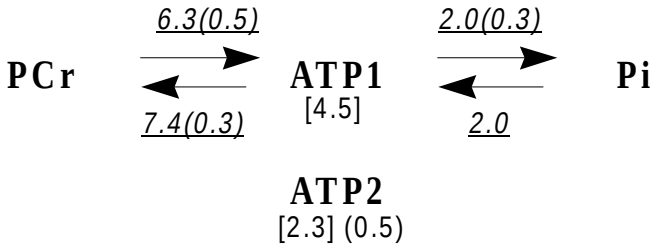
For each diagram the computed fluxes (in $mM.s^{-1}$) are shown in *italics*, the confidence intervals in parenthesis, and the computed concentrations of ATP compartments (in mM) are in brackets.



Ff/Fr = 0.84 T1
PCr = 2.9 s (0.3)
T1 γATP = 0.8 s (0.1)
Number of variables = 5
X^2 = 6.93

Model II: three-site exchange — 2 ATP compartments

Model II additionally takes into account a strict compartmentation of ATP not exchanging through CK as suggested by the results of part I where ATP_2 behaves as an isolated compartment when assessed by inversion transfer under saturation of Pi.

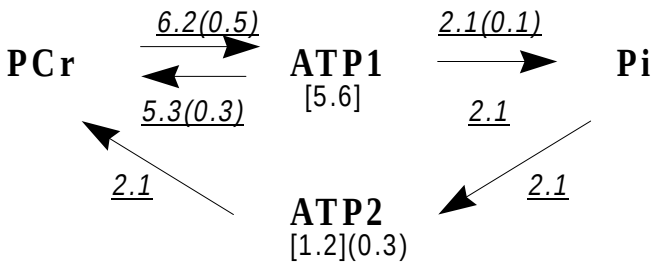


Ff/Fr = 0.86
T1 PCr = 2.5 s (0.2)
T1 γATP_1 = 1.3 s (0.4)
T1 γATP_2 = 0.4 s (0.1)
ATP_2 (% of total ATP) = 33
number of variables = 7
X^2 = 2.26

This model can however be discarded on the basis of studies using ^{18}O labeling [3] which probes net flux of kinases, showing that ultimately about 90% of cellular ATP becomes labeled through kinases.

Model III: three-site exchange — 2 ATP compartments in exchange with PCr

Part I suggested that ATP_2 might be a mitochondrial ATP compartment. Fractionation technique gives access to a compartment which includes both the mitochondrial matrix and the space between the inner and outer membranes. Thus we considered an exchange of ATP_2 with Pi (via ATP synthesis) and with PCr (via the adenine nucleotide translocator and mito CK). This model is an extension of the scheme shown in part I.



Ff/Fr = 0.84
T1 PCr = 3.1 s (0.3)
T1 γATP_1 = 0.7 s (0.1)
T1 γATP_2 = 0.7 s
ATP_2 (%) = 18
number of variables = 6
X^2 = 3.46

Two additional constraints were imposed: T1 γATP_1 = T1 γATP_2 and steady state for ATP_2 (net flux Pi→ATP_2 = ATP_2 → PCr)

The experimental data of the 4 successive inversion protocols and return to equilibrium (eq.) are shown in Fig. 17.1 with the fit of data in this model.

Model IV: three-site — 3 compartments of ATP (and CK)

The myocardium is characterized by a high compartmentation of CK isoforms: 20% of total CK located in the space between inner and outer mitochondrial membrane close to the adenine nucleotide translocase (mito-CK isoform), 25% (MM-CK isoform) located in the vicinity of the ATPases (myofilaments and sarcoplasmic reticulum) thus only 55% is cytosolic. Model IV considers this localization and the resulting compartments of ATP.

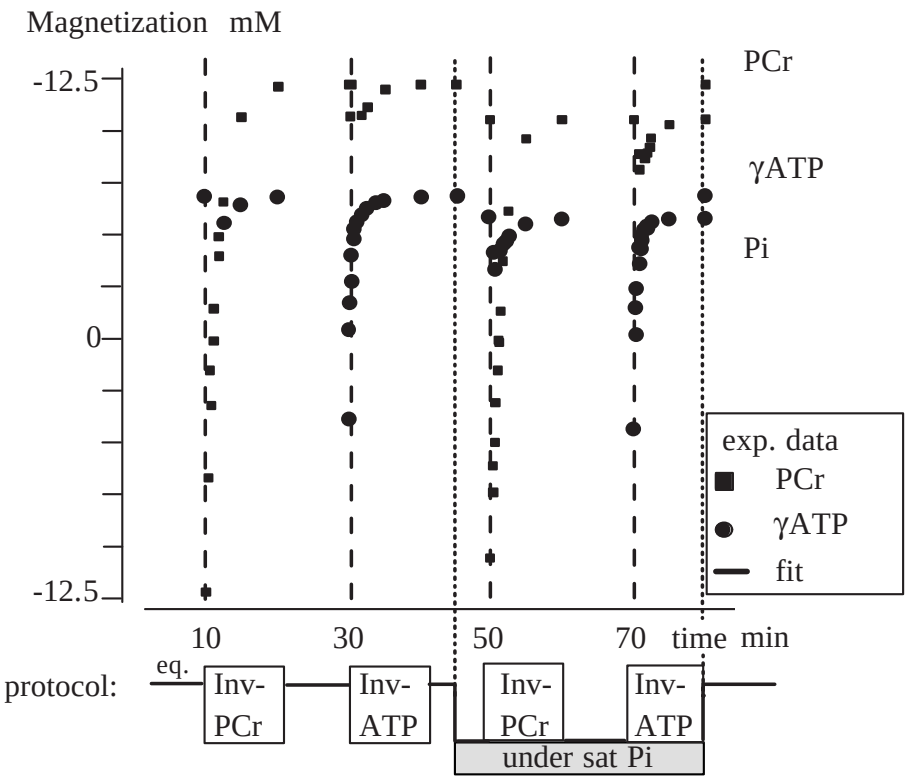
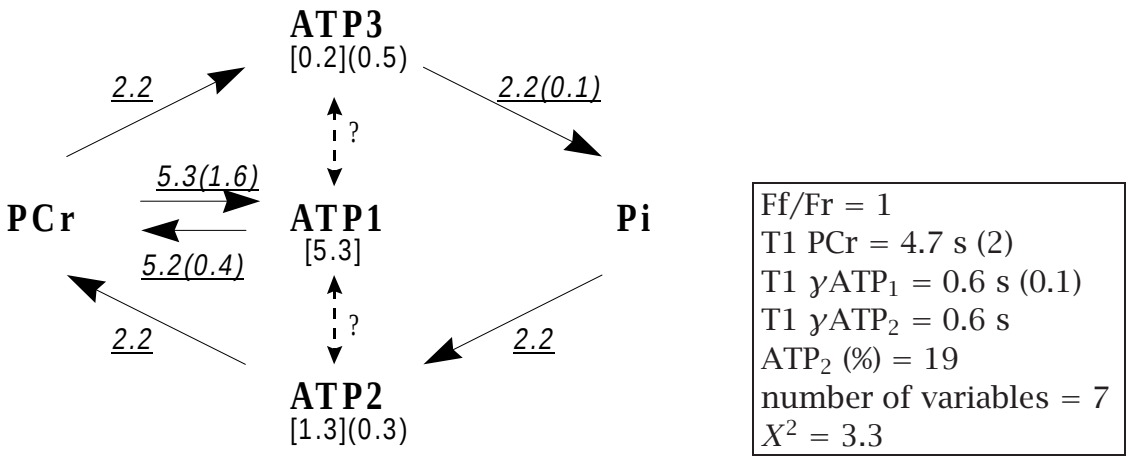


Fig. 17.1 Fit of experimental data to model III data of the 4 successive inversion protocols and return to equilibrium (eq.)



Additional constraints: T1 γATP_1 = T1 γATP_2 and steady state for ATP_2 and ATP_3 (net flux Pi→ATP_2 = ATP_2 → PCr = PCr→ATP_3 = ATP_3 →Pi).

Model IV was the only model leading to equal forward and reverse CK flux. Notice that, as in model III, no information on the diffusion fluxes ATP_2 →ATP_1 and ATP_1 →ATP_3could be obtained with reliable confidence interval. Nevertheless the existence of diffusion fluxes does not affect the result of the model concerning the equality of Ff and Fr since both diffusion fluxes must be identical. In its actual

state, this model corresponds to a strict Cr-PCr-CK shuttle.

Model I can be rejected on the basis of high X^2 and model II from our knowledge of the exchange of ATP in the cell. We can not discriminate between model III and IV on the basis of a better fit of the experimental data of control hearts. However we believe that by applying this analysis to the various experimental conditions which modify ATP synthesis or ATP compartmentation (i.e., the various conditions presented in part I) will help to discriminate between models and might give access to ATP diffusion and bound CK fluxes.

In conclusion this combined analysis offers a mass of information which can not be obtained when using a single protocol of magnetization transfer and might be helpful in our understanding of the complexity of energy transfer flux in the myocardium.

References

1. Brindle, K.M. (1988) *Progress in NMR spectroscopy* **20**, 257–293.
2. Ugurbil, K., Petein M.A., Maiden R., Michurski S.P. and From A.H.L. (1986) *Biochemistry* **25**, 100–108.
3. Zeleznikar R.J. and Goldberg N.D. (1991) *J. Biol. Chem.* **266**, 15110–15119.