

## Evidence for Myocardial ATP Compartmentation from NMR Inversion Transfer Analysis of Creatine Kinase Fluxes

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**ABSTRACT** The interpretation of creatine kinase (CK) flux measured by <sup>31</sup>P NMR magnetization transfer in vivo is complex because of the presence of competing reactions, metabolite compartmentation, and CK isozyme localization. In the isovolumic perfused rat heart, we considered the influence of both ATP compartmentation and ATP-P<sub>i</sub> exchange on the forward ( $F_f$ : PCr → ATP) and reverse ( $F_r$ ) CK fluxes derived from complete analysis of inversion transfer. Although  $F_f$  should equal  $F_r$  because of the steady state, in both protocols when PCr (inv-PCr) or ATP (inv-ATP) was inverted and the contribution of ATP-P<sub>i</sub> was masked by saturation of P<sub>i</sub> (sat-P<sub>i</sub>),  $F_f/F_r$  significantly differed from 1 ( $0.80 \pm 0.06$  or  $1.32 \pm 0.06$ , respectively,  $n = 5$ ). These discrepancies could be explained by a compartment of ATP ( $f_{\text{ATP}}$ ) not involved in CK. Consistently, neglecting ATP compartmentation in the analysis of CK in vitro results in an underestimation of  $F_f/F_r$  for inv-PCr and its overestimation for inv-ATP. Both protocols gave access to  $f_{\text{ATP}}$  if the system was adequately analyzed. The fraction of ATP not involved in CK reaction in a heart performing medium work amounts to 20–33% of cellular ATP. Finally, the data suggest that the effect of sat-P<sub>i</sub> might not result only from the masking of ATP-P<sub>i</sub> exchange.

### INTRODUCTION

The creatine kinase (CK) catalyzes the reversible exchange of high-energy phosphate:



Despite considerable efforts devoted to the study of the flux of this enzyme by <sup>31</sup>P NMR spectroscopy, there is still some controversy about the equivalence of the various magnetization transfer techniques and about the physiological interpretation of the NMR measured CK fluxes. The development of transgenic mice with specific CK isoform knockout has renewed the interest in the analysis of CK flux (Van Dorsten et al., 1998).

The role of different CK isoforms in the myocardium is still under debate: a cytosolic CK working close to equilibrium might account for the replenishment of ATP stores upon ATP utilization and the energy diffusion across the cell. On the other hand, bound CK specifically localized close to sites of energy production and utilization might play a major role in myocardial energy transduction. Nevertheless, even if the bound isoforms have kinetics different from those of the cytosolic CK, the cell is in steady state, and PCr is only metabolized by CK. Therefore, both total CK fluxes (corresponding to the unidirectional flux of production and consumption of ATP by CK) measured by NMR should be equal.

The main problem in the interpretation of a magnetization transfer experiment in vivo arises from the oversimplification of a highly organized cellular system: in other words, the interpretation of data is always model dependent. Most of the work devoted to cardiac CK flux has been performed by saturation transfer technique (ST). Consistent data have been obtained for the determination of the forward CK flux PCr → ATP ( $F_f$ ), whereas a large range of values has been reported in the literature for the reverse CK flux ATP → PCr ( $F_r$ ) (see Table 1). When a discrepancy between  $F_f$  and  $F_r$  was observed, two main alternative hypotheses were proposed. First, the two-site model of CK reaction has been recognized as an oversimplification of the system: ATP is involved in other exchanges, mainly with P<sub>i</sub> (Ugurbil et al., 1986; Spencer et al., 1988), but also with glycolytic metabolites (Matthews et al., 1982; Brindle, 1988). The second hypothesis is the presence of an ATP compartmentation initially suggested by Nunnally and Hollis (1979). The discrepancy between fluxes, which increases with cardiac performance (Koretsky et al., 1985), was further proposed to result from the presence of an additional exchange between either PCr and a small NMR-visible ATP pool (Koretsky et al., 1985, 1986) or between PCr and a nonvisible nonsaturated ATP compartment (Zahler et al., 1987; Zahler and Ingwall, 1992).

Inversion transfer (IT) has mainly been performed in muscle by analyzing the initial rate of recovery, which allowed a simplification of the mathematical analysis and a reduction in experimental time (Hsieh and Balaban, 1988; McFarland et al., 1994). However, the complete analysis of the evolution of the magnetization of the inverted and noninverted species permits the determination in the same experiment of both  $F_f$  and  $F_r$  (Led and Gesmar, 1982). Furthermore, data can then be analyzed without imposing the equilibrium of CK on the system, while this simplifica-

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**TABLE 1** Comparison of published values of the  $F_f/F_r$  ratio

Experiment	Substrate	Organ	$F_f/F_r$	Reference	
Saturation	SSST	Acetate*	Heart	2.1	Matthews et al. (1982)
		Pyruvate <sup>†</sup>	Heart	1.8	Ugurbil et al. (1986)
		Pyruvate	Heart	3.0	Zweier and Jacobus (1987)
		Glucose*	Heart	1.2	Matthews et al. (1982)
		Glucose	Heart	1.0	Degani et al. (1985)
		Glucose	Heart	1.1	Ugurbil et al. (1986)
		Glucose	Heart	1.2	Zweier and Jacobus (1987)
		In vivo	Heart	1.6	Koretsky et al. (1986)
		In vivo	Skeletal muscle	2.0	Hsieh and Balaban (1988)
		TDST	Acetate	Heart	1.6
Heart	1.4			Bittl and Ingwall (1985)	
Heart	1.5			Spencer et al. (1988)	
Heart	1.1			Bittl et al. (1987)	
Skeletal muscle	1.1			Bittl et al. (1987)	
Inversion	Initial slope	Pyruvate	Skeletal muscle	1.0	McFarland et al. (1994)
		In vivo	Skeletal muscle	0.9	Hsieh and Balaban (1988)
		In vivo	Brain	1.5	Mora et al. (1992)
	Full analysis	Glucose	Heart	1.0	Degani et al. (1985)
			Brain	1.2	Degani et al. (1987)
			Brain	0.6	Mora et al. (1992)

SSST, Steady-state saturation transfer; TDST, time-dependent saturation transfer.

\*In the absence of contractile activity  $F_f/F_r$  is 1 in both glucose or acetate KCl arrested hearts.

<sup>†</sup>Both pyruvate and glucose present.

This table emphasizes the influence of various factors on the determination of  $F_f/F_r$ : the magnetization transfer protocol, the substrate used, and the work performed.

tion of the Bloch equations is usually applied in the analysis of ST. Such analysis of IT offers a definitive advantage over the other magnetization transfer techniques.

Using IT, we found unequal CK fluxes in myocardium. To interpret these data, we analyzed the consequences for the measured CK fluxes of the presence of an ATP pool not involved in the CK reaction (or in slow exchange), as earlier suggested by Meyer et al. (1982). We considered total cellular ATP and PCr to be fully NMR visible and disturbed by the inversion procedure. The Bloch equations were modified to take into account this additional compartment of ATP, in both cases of an inversion of PCr (inv-PCr) and of  $\gamma$ ATP (inv- $\gamma$ ATP) (see section Theory).

Our aim was to explore the capacity of a full time-course analysis of inversion transfer to reveal experimentally both the specific contributions of ATP compartmentation and ATP- $P_i$  exchange in the CK fluxes of a perfused heart. Based on in vitro simulation we show that the presence of an ATP compartment (not exchanging with PCr) is consistent with the flux discrepancies observed in situ. Moreover, under conditions designed to mask the influence of ATP- $P_i$  exchange, this ATP compartment could be independently quantified in both inv-PCr and inv- $\gamma$ ATP protocols.

## THEORY

Classical Bloch equations for chemical exchange (McConnell, 1958) had to be modified to account first for the

fraction of ATP not involved in the CK reaction,  $f_{\text{ATP}}$ , and second for the involvement of ATP in other reactions. In the following sections we refer to a phosphorus species as a “site” and a kinetic compartment as a “compartment.”

### Model I: two-site two-compartment analysis: basic model

The CK reaction is considered as a two-site exchange of the  $^{31}\text{P}$  nucleus:



where  $k_f$  and  $k_r$  are, respectively, the forward and reverse pseudo-first-order rate constants of the reaction. Considering the magnetization at equilibrium,  $M_{\text{PCr}}^{\infty}$  and  $M_{\gamma\text{ATP}}^{\infty}$ , the forward flux  $\text{PCr} \rightarrow \text{ATP}$  and the reverse flux  $\text{ATP} \rightarrow \text{PCr}$  are given respectively by

$$F_f = k_f \cdot M_{\text{PCr}}^{\infty} \quad (1)$$

$$F_r = k_r \cdot M_{\gamma\text{ATP}}^{\infty} \quad (2)$$

The evolution of magnetization is described by the modified Bloch equations (Ugurbil, 1985), where  $T_{1\gamma\text{ATP}}$ ,  $T_{1\text{PCr}}$  are the intrinsic relaxation parameters:

$$\begin{aligned} dM_{\text{PCr}}/dt = & -(k_f + 1/T_{1\text{PCr}})(M_{\text{PCr}} - M_{\text{PCr}}^{\infty}) \\ & + k_r(M_{\gamma\text{ATP}} - M_{\gamma\text{ATP}}^{\infty}) \end{aligned} \quad (3)$$

$$\begin{aligned} dM_{\gamma\text{ATP}}/dt = & -(k_r + 1/T_{1\gamma\text{ATP}})(M_{\gamma\text{ATP}} - M_{\gamma\text{ATP}}^{\infty}) \\ & + k_f(M_{\text{PCr}} - M_{\text{PCr}}^{\infty}) \end{aligned} \quad (4)$$

The solutions of this system depend on the protocol of magnetic perturbation.

#### Time-dependent saturation transfer

Upon full saturation of  $\gamma\text{ATP}$  ( $M_{\gamma\text{ATP}} = 0$ ),  $M_{\text{PCr}}$  decreases because of chemical exchange between PCr and  $\gamma\text{ATP}$ . Considering the equilibrium of CK,  $k_f \cdot M_{\text{PCr}}^{\infty} = k_r \cdot M_{\gamma\text{ATP}}^{\infty}$ , Eq. 3, which describes the time evolution of  $M_{\text{PCr}}$  becomes

$$M_{\text{PCr}}(t) = M_{\text{PCr}}^{\text{ss}} + (M_{\text{PCr}}^{\infty} - M_{\text{PCr}}^{\text{ss}})\exp[-(k_f + 1/T_{1\text{PCr}})t] \quad (5)$$

where  $M_{\text{PCr}}^{\text{ss}} = M_{\text{PCr}}^{\infty}/(1 + k_f T_{1\text{PCr}})$ . From this experiment  $k_f$  and  $T_{1\text{PCr}}$  may be derived. Forward flux  $F_f$  is determined from the accurate knowledge of  $M_{\text{PCr}}^{\infty}$  and Eq. 1.

#### Inversion transfer

After a selective inversion of either PCr or  $\gamma\text{ATP}$ , the chemical exchange occurs between the two magnetizations. In this case, the solutions of Eq. 3 and 4 are given by the general expression (Led and Gesmar, 1982):

$$M_{\text{PCr}}(t) = M_{\text{PCr}}^{\infty} + C_1 \exp \lambda_1 t + C_2 \exp \lambda_2 t \quad (6)$$

$$M_{\gamma\text{ATP}}(t) = M_{\gamma\text{ATP}}^{\infty} + C_3 \exp \lambda_1 t + C_4 \exp \lambda_2 t \quad (7)$$

with

$$\lambda_1 = 1/2\{-(k_{1f} + k_{1r}) + [(k_{1f} - k_{1r})^2 + 4k_f k_r]^{1/2}\}$$

$$\lambda_2 = 1/2\{-(k_{1f} + k_{1r}) - [(k_{1f} - k_{1r})^2 + 4k_f k_r]^{1/2}\}$$

where

$$k_{1f} = k_f + 1/T_{1\text{PCr}} \quad k_{1r} = k_r + 1/T_{1\gamma\text{ATP}}$$

and

$$C_1 = \{(\lambda_2 + k_{1f})(M_{\text{PCr}}^{\infty} - M_{\text{PCr}}(0)) - k_r(M_{\gamma\text{ATP}}^{\infty} - M_{\gamma\text{ATP}}(0))\}/(\lambda_1 - \lambda_2)$$

$$C_2 = \{-(\lambda_1 + k_{1f})(M_{\text{PCr}}^{\infty} - M_{\text{PCr}}(0)) + k_r(M_{\gamma\text{ATP}}^{\infty} - M_{\gamma\text{ATP}}(0))\}/(\lambda_1 - \lambda_2)$$

$$C_3 = \{-k_f(M_{\text{PCr}}^{\infty} - M_{\text{PCr}}(0)) - (\lambda_1 + k_{1f}) \cdot (M_{\gamma\text{ATP}}^{\infty} - M_{\gamma\text{ATP}}(0))\}/(\lambda_1 - \lambda_2)$$

$$C_4 = \{k_f(M_{\text{PCr}}^{\infty} - M_{\text{PCr}}(0)) + (\lambda_2 + k_{1f}) \cdot (M_{\gamma\text{ATP}}^{\infty} - M_{\gamma\text{ATP}}(0))\}/(\lambda_1 - \lambda_2)$$

From inversion of PCr, or of  $\gamma\text{ATP}$ , the four parameters of the reaction,  $k_f$ ,  $k_r$ ,  $T_{1\text{PCr}}$ ,  $T_{1\gamma\text{ATP}}$  may be computed inde-

pendently, and both  $F_f$  and  $F_r$  fluxes calculated from Eqs. 1. and 2.

### Model II: two-site three-compartment analysis: modification accounting for a compartment of ATP not involved in CK



In the case of two pools of ATP, one pool,  $\text{ATP}_1 = (1 - f_{\text{ATP}}) \cdot \text{ATP}$ , in fast exchange with PCr, and a second,  $\text{ATP}_2 = f_{\text{ATP}} \cdot \text{ATP}$ , not exchanging (or in slow exchange) with PCr, the evolution of each pools must be considered separately; the time evolution of  $\text{ATP}_1$  is described by Eq. 4, and that of  $\text{ATP}_2$  by

$$dM_{\gamma\text{ATP}_2}/dt = -(M_{\gamma\text{ATP}_2} - M_{\gamma\text{ATP}_2}^{\infty})/T_{1\gamma\text{ATP}_2} \quad (8)$$

The solutions of this new system (Eqs. 3, 4, and 8) depend on the type of magnetization transfer protocol. In time-dependent saturation transfer (TDST), neglecting ATP compartmentation will not affect  $F_f$  (measured by saturation of  $\gamma\text{ATP}$ ).  $F_r$ , measured by saturation of PCr, will also be correct, as pointed out earlier (Meyer et al., 1982; Spencer et al., 1988): the underestimation of  $k_r$  (by  $1 - f_{\text{ATP}}$ ) is indeed exactly compensated for by an overestimation of the ATP content. However, in IT, the presence of an ATP compartment will affect the analysis of both PCr and  $\gamma\text{ATP}$  inversion in a different way.

#### Inversion of PCr

Magnetization of  $\text{ATP}_2$ , not initially disturbed, thus is constant during the experiment:  $dM_{\gamma\text{ATP}_2}/dt = 0$ . The evolution of ATP magnetization,  $M_{\gamma\text{ATP}}(t)$ , depends only on  $M_{\gamma\text{ATP}_1}$  and is still described by Eq. 7; that of PCr,  $M_{\text{PCr}}(t)$ , follows Eq. 6.  $F_f$  is still given by Eq. 1. However, because only the sum  $M_{\gamma\text{ATP}} = M_{\gamma\text{ATP}_1} + M_{\gamma\text{ATP}_2}$  is experimentally observed, only an apparent reverse flux ( $k_r \cdot M_{\gamma\text{ATP}}^{\infty}$ ) is accessible. The steady state is described by  $k_f \cdot M_{\text{PCr}}^{\infty} = k_r \cdot M_{\gamma\text{ATP}}^{\infty}$ . Because  $M_{\gamma\text{ATP}}^{\infty} = M_{\gamma\text{ATP}_1}^{\infty}/(1 - f_{\text{ATP}})$ , the ratio  $F_f/F_r$  equals  $(1 - f_{\text{ATP}})$ . Therefore, in an inv-PCr protocol, correct kinetic parameters can be obtained, and the measured  $F_f/F_r$  directly quantifies the fraction of ATP not involved in the CK reaction.

#### Inversion of $\gamma\text{ATP}$

In this protocol, both  $\gamma\text{ATP}_1$  and  $\gamma\text{ATP}_2$  are inverted:  $M_{\text{PCr}}(t)$  and  $M_{\gamma\text{ATP}_1}(t)$  are still described by Eqs. 6 and 7, but the evolution of  $M_{\gamma\text{ATP}}$  must now include the evolution of  $M_{\gamma\text{ATP}_2}$ , described by

$$M_{\gamma\text{ATP}_2}(t) = M_{\gamma\text{ATP}_2}^{\infty} + (M_{\gamma\text{ATP}_2}(0) - M_{\gamma\text{ATP}_2}^{\infty})\exp[-t/T_{1\gamma\text{ATP}_2}] \quad (9)$$

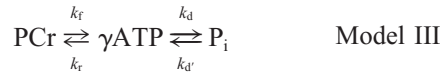
The time evolution of  $M_{\gamma\text{ATP}}$  is thus described by a triple exponential:

$$M_{\gamma\text{ATP}}(t) = M_{\gamma\text{ATP}}^{\infty} + C_3 \exp \lambda_1 t + C_4 \exp \lambda_2 t + (M_{\gamma\text{ATP}_2}(0) - M_{\gamma\text{ATP}_2}^{\infty}) \exp - (t/T_{1\gamma\text{ATP}_2}) \quad (10)$$

The parameters of the CK reaction, as well as  $T_{1\gamma\text{ATP}_2}$ , can be obtained by fitting  $M_{\gamma\text{ATP}}(t)$  by Eq. 10 and  $M_{\text{PCr}}(t)$  by Eq. 6. Again,  $F_f/F_r$  gives  $(1 - f_{\text{ATP}})$ . Neglecting the contribution of Eq. 9 and performing a two-site analysis will induce errors in all parameters and thus in both fluxes. Notice that in this case, the ratio  $F_f/F_r$  will be overestimated.

### Model III: three-site three-compartment analysis: modification accounting for the implication of ATP in other exchanges

In contrast to PCr which is only metabolized by CK in the cell, ATP is involved in many other cellular reactions. Because of the high activity of ATP synthesis and hydrolysis in muscle, the ATP- $P_i$  exchange has been recognized as a possible main source of artifact in the determination of CK flux: a three-site three-compartment exchange model has been proposed (Ugurbil et al., 1986; Spencer et al., 1988):



In this frame, elimination of the effect of  $P_i \rightarrow \text{ATP}$  exchange by a continuous saturation of  $P_i$  (sat- $P_i$ ) will reduce the model to a two-site two-compartment exchange (model I) described by a new set of equations analogous in form to Eqs. 3 and 4 (for a complete description, see Ugurbil, 1985):

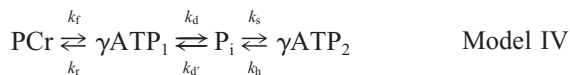
$$dM_{\text{PCr}}/dt = -(k_f + 1/T_{\text{PCr}})(M_{\text{PCr}} - M_{\text{PCr}}^*) + k_r(M_{\gamma\text{ATP}} - M_{\gamma\text{ATP}}^*) \quad (11)$$

$$dM_{\gamma\text{ATP}}/dt = -(k_r + 1/T_{1\gamma\text{ATP}}^*)(M_{\gamma\text{ATP}} - M_{\gamma\text{ATP}}^*) + k_f(M_{\text{PCr}} - M_{\text{PCr}}^*) \quad (12)$$

where  $M_{\text{PCr}}^*$  and  $M_{\gamma\text{ATP}}^*$  are the steady-state magnetization under sat- $P_i$ , and  $1/T_{1\gamma\text{ATP}}^* = k_d + 1/T_{1\gamma\text{ATP}}$

### Model IV: three-site four-compartment analysis: modification accounting for both ATP compartmentalization and the implication of ATP in other exchanges

In the case of ATP compartmentation,



A complete description of the system in the absence of  $P_i$  saturation is given in the Appendix. With the saturation of  $P_i$  this model reduces to model II with modified intrinsic relaxation parameters, if the only consequence of sat- $P_i$  is to mask the influence of the  $P_i \rightarrow \text{ATP}$  exchange. Equation 12 applies to the evolution of  $M_{\gamma\text{ATP}_1}$ . The time evolution of  $M_{\gamma\text{ATP}_2}$  is described as previously by model II and Eq. 8. Notice that the determination of  $F_f$  by the saturation of  $\gamma\text{ATP}$  is the only experimental protocol that is not influenced by the presence of a  $P_i \leftrightarrow \text{ATP}$  exchange and can thus be used as a reference for in vivo flux determination.

In summary, this formalism describes how to take into account the presence of an ATP compartment in the analysis of both inversion of PCr and  $\gamma\text{ATP}$  and shows that both protocols can independently quantify an ATP compartment.

## MATERIALS AND METHODS

### Enzyme solutions

Measurement of CK flux in vitro was performed using two concentric tubes (OD tube 1 = 8 mm, tube 2 = 20 mm). Both tubes contained solutions, the initial composition of which was (in mM) 10 Cr, 5.7 Mg, 1 dithiothreitol, 0.5 EGTA, 100 HEPES, and 15%  $\text{D}_2\text{O}$ . ATP (5 mM), PCr (10 mM), and rabbit muscle creatine kinase (Boehringer) at a concentration of 800 IU/ml were added to tube 1. Variable concentrations of ATP ranging from 0 to 1.2 mM (corresponding to a fraction of ATP<sub>2</sub> not involved in CK reaction ranging from 0 to 50% of the total ATP) were added to tube 2, which did not contain CK.  $T_{1\gamma\text{ATP}_2}$  stays constant and similar to  $T_{1\gamma\text{ATP}_1}$  in this protocol. The pH of the final solutions was adjusted with acetic acid at 7.1 at 30°C. The flux measurement was performed at 30°C. To evaluate the possibility of measuring  $T_{1\gamma\text{ATP}_2}$  in our in vitro system, a second series of experiments was performed in which tube 2 contained a fixed ATP concentration of 1 mM (corresponding to 45% of the total ATP), but its relaxation properties were modified by replacing  $\text{H}_2\text{O}$  in the solution with various amounts of glycerol (from 15 to 70%) to change the viscosity of the solution.

### Isolated perfused rat hearts

Animal experimentation was performed in accordance with the Helsinki Accords for Humane Treatment of Animals during Experimentation. Wistar male rats (350–450 g) were anesthetized with ethyl carbamate (2 g/kg), and hearts were perfused by the Langendorff technique at a constant flow of  $13.5 \text{ ml}\cdot\text{min}^{-1}$  as previously described (Hoerter et al., 1988). Briefly, a latex balloon was inserted into the left ventricle (LV) and inflated with  $\text{D}_2\text{O}$  to isovolumic conditions of work. Mean coronary pressure, LV systolic pressure (LVP), end diastolic pressure (EDP), and heart spontaneous frequency were continuously monitored on a computer (Compaq) via Statham gauges. The rate pressure product (RPP in  $10^4 \text{ mmHg}\cdot\text{beats}\cdot\text{min}^{-1}$ ) was used as an index of contractility reflecting the energetic demand. The perfusion solution contained (in mM) 124 NaCl, 6 KCl, 1.8  $\text{CaCl}_2$ , 1  $\text{MgSO}_4$ , 1.1 mannitol, 10 Na-acetate, and 20 HEPES and was oxygenated with 100%  $\text{O}_2$ . The  $\text{pH}_0$  was adjusted to 7.35 at 36.5°C. Hearts were freeze clamped at the end of the experiment to measure their ATP, PCr, and Cr contents (in  $\text{nmol}\cdot\text{mg}\cdot\text{prot.}^{-1}$ ) (Hoerter et al., 1988). These values were used to calculate the metabolite concentrations during magnetization transfer. CK flux was expressed in  $\text{mM}\cdot\text{s}^{-1}$  (assuming cytosolic  $\text{H}_2\text{O}$  volume =  $2.72 \mu\text{l}\cdot\text{mg}\cdot\text{prot.}^{-1}$ ).



## NMR

$^{31}\text{P}$  NMR spectra were acquired on a Innova Varian with a 9.4-T wide-bore magnet in 8-mm- and 20-mm-diameter tubes for the *in vitro* analysis and in a 20-mm tube for the heart. We used a pulse angle of  $80^\circ$ , 4 K data point acquisition, a spectral width of 10,000 Hz, an acquisition time of 0.205 s, a repetition time of 10 s, zero filling to 8 K, and line broadening of 20 Hz. For all flux measurements, 32-scan spectra were acquired by blocs of eight scans cycling four times through the whole protocol. For the heart experiment, homogeneity was performed on the heart water, the frequency was locked on  $\text{D}_2\text{O}$  contained in the LV balloon, and the  $360^\circ$  pulse duration was measured on the  $\gamma\text{ATP}$  peak. After 20 min of equilibration, four partially saturated spectra (repetition time 2 s, number of scans 32) were acquired. The stability of the preparation was checked by comparing fully relaxed control spectra (repetition time 10 s) acquired before and after the magnetization transfer experiment; any heart showing more than 10% variation in its metabolite content was discarded. These controls were also used to check the complete recovery of magnetization after 10 s of mixing time during inversion protocols. In four hearts TDST of  $\gamma\text{ATP}$  (Bittl and Ingwall, 1985) was performed as a reference for the determination of forward CK flux. Eight spectra were acquired with a duration of  $\gamma\text{ATP}$  saturation ranging from 0 to 9 s (a 9-s irradiation at mirror frequency was used to test radiation spillover). The delays between pulses were adjusted to achieve a constant rate of recurrence of 10 s in each spectrum. Inversion recovery (IR) was used to measure  $T_{1\gamma\text{ATP}}$  *in vitro* (eight spectra, 16 scans, delays ranging from 0 to 10 s). The inversion transfer (IT) experiments were performed with a pulse sequence consisting of a frequency-selective sinc pulse (15 ms, bandwidth 300 Hz), a variable mixing time delay (0.2–10 s), a  $80^\circ$  read pulse, and a fixed 10-s delay to allow full relaxation before the next inversion. Each inversion transfer experiment required 26 spectra *in vitro* (total time 75 min) and 13 spectra for the heart (total time 75 min). Five hearts were used for each protocol of inversion of PCr (inv-PCr) or  $\gamma\text{ATP}$  (inv- $\gamma\text{ATP}$ ). The effect of a shorter repetition time of 4 s was additionally checked in four hearts (inv-PCr,  $n = 2$ ; inv- $\gamma\text{ATP}$ ,  $n = 2$ ). In 10 other hearts inv- $\gamma\text{ATP}$  or inv-PCr was performed under continuous saturation of  $\text{P}_i$ . Although off-resonance effects of a sat- $\text{P}_i$  protocol on PCr could not be checked directly by mirror irradiation, we considered as negligible the spillover of  $\text{P}_i$  saturation because similar irradiation applied at 2.4 ppm from PCr (chemical shift of  $\text{P}_i = 4.86$  ppm from PCr) resulted in a  $\sim 3\%$  decrease in PCr.

## Analysis

In TDST protocols, the exponential decay of  $\gamma\text{ATP}$  magnetization, estimated by peak areas, was analyzed to determine in the same experiment  $T_{1\text{PCr}}$  and  $k_f$  (Bittl and Ingwall, 1985). In IT experiments, the adjustment of experimental data with theoretical expression was performed using either the peak areas or the product of peak amplitudes by  $\alpha$ , as described by Led and Gesmar (1982), which should address the total NMR visible cellular metabolites.  $\alpha$ , the average ratio of peak area to amplitude, was measured for each heart (mean value  $0.61 \pm 0.02$  and  $1.50 \pm 0.03$ ,  $n = 10$ , for inv-PCr and inv- $\gamma\text{ATP}$ , respectively). The two analyses gave similar results; only the latter is shown here because it results in smaller data scattering. The evolution of the inverted and noninverted species was fitted to the solutions of Bloch equations, using the Levenberg-Marquardt method. Equations 6 and 7 were used for the two-site two-compartment analysis of inv-PCr and inv- $\gamma\text{ATP}$  protocol. The adjustable parameters in this analysis were  $k_f$ ,  $k_r$ ,  $T_{1\text{PCr}}$ , and the magnetizations of PCr and ATP at  $t = 0$  and  $t = \infty$ . Two-site three-compartment analysis of the inv-ATP experiment under saturation of  $\text{P}_i$  had to be performed by a three-exponential fit of  $M_{\gamma\text{ATP}}$  using Eq. 10. However, because of the number of unknown parameters, the confidence interval for the parameters derived from this analysis was high both *in vitro* and in the myocardium. To improve this analysis,  $F_f$  and  $T_{1\text{PCr}}$  measured in the inv-PCr experiment were imposed as fixed parameters (for justification see the Results).

All data were expressed as mean  $\pm$  SE. Differences between groups were analyzed by *t*-test, paired *t*-test, or variance analysis and Student-Newman-Keuls test.

## RESULTS

### *In vitro* validation of the model

*Variation of fraction of the ATP ( $f_{\text{ATP}}$ ) that does not participate in the CK reaction*

To test the model *in vitro*, the fraction  $f_{\text{ATP}}$  of ATP not involved in the CK reaction was first varied from 0 to 0.5, while  $T_{1\gamma\text{ATP}2}$  was kept constant (and equal to  $T_{1\gamma\text{ATP}1}$ ).

*Inversion of PCr.* The parameters of the CK reaction  $k_f$ ,  $k_r$ ,  $T_{1\text{PCr}}$ , and  $T_{1\gamma\text{ATP}1}$  were first measured in the absence of compartmentalization ( $f_{\text{ATP}} = 0$ ). As expected from the *in vitro* CK kinetics,  $F_f$  was equal to  $F_r$ . With the increase in  $f_{\text{ATP}}$ , the values of  $k_f$ ,  $k_r$ ,  $T_{1\text{PCr}}$ , and  $T_{1\gamma\text{ATP}1}$  remained constant as predicted by the theoretical development; their mean values were  $k_f = 0.30 \pm 0.01 \text{ s}^{-1}$ ,  $k_r = 0.57 \pm 0.02 \text{ s}^{-1}$ ,  $T_{1\text{PCr}} = 4.2 \pm 0.3 \text{ s}$ , and  $T_{1\gamma\text{ATP}1} = 2.4 \pm 0.2 \text{ s}$ . Fig. 1 *a* shows the evolution of the measured  $F_f$  and  $F_r$  as a function of  $f_{\text{ATP}}$  as well as their theoretical prediction. The  $F_f$  value remained constant ( $1.00 \pm 0.04 \text{ MU}\cdot\text{s}^{-1}$ ). The observed increase in  $F_r$  (from 0.98 to  $1.96 \text{ MU}\cdot\text{s}^{-1}$ ) was due solely to the overestimation of the ATP used in the flux calculation. As a result, the ratio  $F_f/F_r$  decreased from 1 to 0.5.  $F_f/F_r$  was a direct measure of  $(1 - f_{\text{ATP}})$  as expected from the theory. Indeed, an excellent correlation was observed between the size of the ATP compartment imposed experimentally and its value as estimated by inv-PCr ( $r^2 = 0.97$ ).

*Inversion  $\gamma\text{ATP}$  protocol.* Likewise, in the absence of ATP compartmentation ( $f_{\text{ATP}} = 0$ ),  $F_f$  and  $F_r$  were equal. When  $f_{\text{ATP}}$  increased, the two-site three-compartment analysis correctly estimated  $k_r$  ( $0.56 \pm 0.04 \text{ s}^{-1}$ ),  $T_{1\gamma\text{ATP}1}$  ( $2.6 \pm 0.4 \text{ s}$ ), and  $T_{1\gamma\text{ATP}2}$  ( $2.5 \pm 0.5 \text{ s}$ ). The fraction  $f_{\text{ATP}}$  extracted from this analysis was well correlated with the size of the ATP compartment ( $r^2 = 0.96$ ; not shown). Neglecting ATP compartmentation (i.e., analyzing the data by the two-site two-compartment model I) did not allow a correct determination of the rate constants. As the fraction of ATP increased (Fig. 1 *b*), a rise was observed both in  $k_f$  (from  $0.28 \text{ s}^{-1}$  to  $0.73 \text{ s}^{-1}$ ) and in  $F_f$  (from 1 to  $1.77 \text{ MU}\cdot\text{s}^{-1}$ ). The rate constant  $k_r$  decreased from  $0.62 \text{ s}^{-1}$  to  $0.26 \text{ s}^{-1}$ . One should notice that in this analysis, no significant change could be experimentally detected in  $F_r$  (mean  $F_r = 0.98 \pm 0.07 \text{ MU}\cdot\text{s}^{-1}$ ). This result suggests that the error made by neglecting  $f_{\text{ATP}}$  in the analysis was balanced by an underestimation of  $k_r$ ; i.e., the error in  $k_r$  can be roughly estimated as  $(1 - f_{\text{ATP}})$ . The ratio  $F_f/F_r$  increased from 1 to  $1.87 \text{ MU}\cdot\text{s}^{-1}$ . Again, Scheme II was adequate to predict the measured  $F_f$  and  $F_r$  in inv- $\gamma\text{ATP}$ . No change were found in the values of  $T_{1\text{PCr}}$  and  $T_{1\gamma\text{ATP}1}$  ( $3.9 \pm 0.4 \text{ s}$  and  $2.4 \pm 0.2 \text{ s}$ , respectively). Thus, in the case of ATP

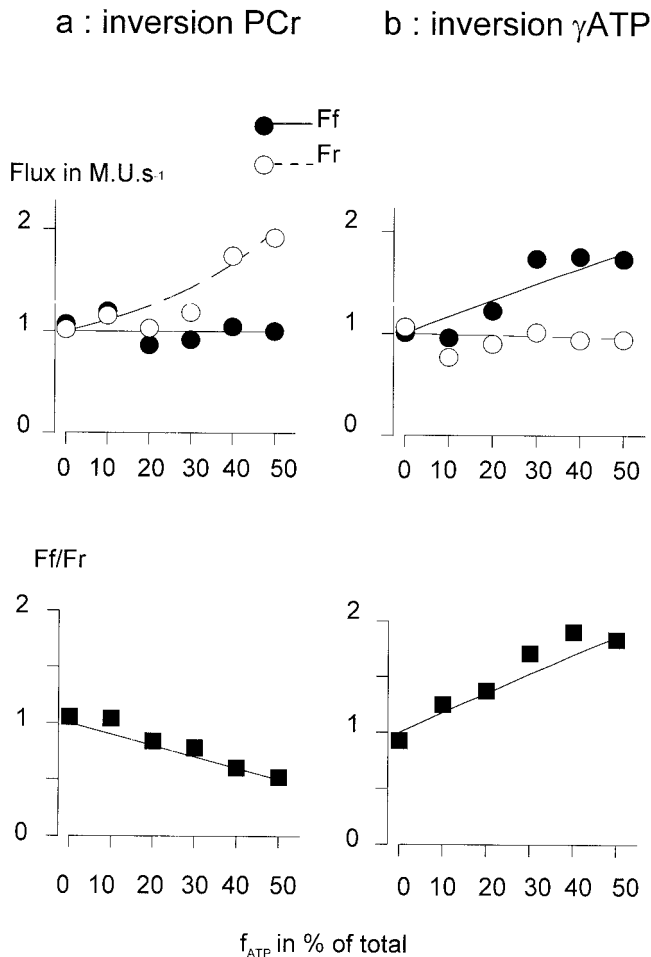


FIGURE 1 Influence of the presence of an ATP compartment on the in vitro determination of CK flux parameters by inversion transfer protocols. (a) Protocol of inversion of PCr. (b) Protocol of inversion of  $\gamma$ ATP. Forward and reverse flux (top) as well as  $F_f/F_r$  ratio (bottom) were expressed as a function of ATP<sub>2</sub> (as a percentage of total ATP) when the fraction of ATP not involved in CK (in tube<sub>2</sub>) was increased. Fluxes (shown in symbols) were computed by fitting experimental data to Eqs. 6 and 7. The continuous lines were obtained by fitting Eqs. 6 and 7 to kinetics reconstructed from the known fraction  $f$  in Eqs. 6 and 11. Input parameters:  $k_f = 0.25 \text{ s}^{-1}$ ,  $k_r = 0.5 \text{ s}^{-1}$ ,  $T_{\text{PCr}} = 3.5 \text{ s}$ ,  $T_{1\gamma\text{ATP}1} = T_{1\gamma\text{ATP}2} = 2.5 \text{ s}$ . Neglecting the ATP compartmentalization in the analysis resulted in errors in flux determination and in  $F_f/F_r$ .

compartmentalization, an analysis using model I generated errors in the determination of the rate constants, of the forward flux, and of the ratio  $F_f/F_r$ , but provided a correct estimation of the reverse CK flux.

#### Variation of $T_{1\gamma\text{ATP}2}$ for a constant fraction $f_{\text{ATP}}$

As shown above, the two-site three-compartment analysis (model II) theoretically allows an estimation of  $T_{1\gamma\text{ATP}2}$ . To test the sensitivity of this determination,  $T_{1\gamma\text{ATP}2}$  was modified by increasing the viscosity of solution with glycerol,

keeping constant the fraction of ATP not involved in CK reaction ( $f_{\text{ATP}} = 0.45$ ). Because of increased viscosity,  $T_{1\gamma\text{ATP}2}$  decreased from 2.5 to 0.8 s, as measured by IR in the absence of CK. The values of  $T_{1\gamma\text{ATP}2}$  obtained from the inv- $\gamma$ ATP protocol were in good correlation ( $r^2 = 0.94$ ,  $n = 5$ ) with those measured by IR.

In conclusion, these in vitro data provided an experimental validation of the theoretical analysis used and showed that the presence of an ATP compartment,  $f_{\text{ATP}}$ , not involved in the CK reaction disturbed the measured kinetics in IT experiments in a predictable manner. In the case of this simple model, accurate estimation of  $f_{\text{ATP}}$  was obtained with inv-PCr. In the inv-ATP protocol, a two-site two-compartment analysis correctly measured  $F_r$  despite errors in all rate constants. The two-site three-compartment analysis, in addition to leading to a correct estimation of all kinetic parameters, provided an independent estimation of  $f_{\text{ATP}}$  and a measure of the intrinsic longitudinal relaxation of ATP in the compartment.

## Application to the perfused rat heart

### Characteristics of the hearts

In the various magnetization protocols hearts developed similar contractile performances; the mean pooled values were LVP =  $148 \pm 4 \text{ mm Hg}$ , frequency =  $271 \pm 6 \text{ beats} \cdot \text{min}^{-1}$  and rate pressure product =  $4.0 \pm 0.1 \times 10^4 \text{ mmHg} \cdot \text{beats} \cdot \text{min}^{-1}$  ( $n = 24$ ). No significant rise in end diastolic pressure occurred during the experiment. Metabolite concentrations during the magnetization transfer period were similar for all inversion protocols; the pooled values were ATP =  $6.8 \pm 0.3 \text{ mM}$ , PCr =  $12.3 \pm 0.3 \text{ mM}$ ,  $P_i = 2.9 \pm 0.2 \text{ mM}$ ,  $\text{pH}_i = 7.09 \pm 0.01$  (PCr to ATP ratio =  $1.96 \pm 0.09$ ). Creatine was  $11.6 \pm 0.3 \text{ mM}$  and free ADP assuming CK equilibrium was  $47 \pm 2 \mu\text{M}$  ( $n = 24$ ).

The parameters of the forward CK flux measured by time-dependent saturation of  $\gamma$ ATP are shown in Table 2. Forward CK flux was  $1.02 \pm 0.04 \text{ MU} \cdot \text{s}^{-1}$  ( $7.3 \pm 0.3 \text{ mM} \cdot \text{s}^{-1}$ ), and  $T_{\text{PCr}}$  was  $3.2 \pm 0.3 \text{ s}$  ( $n = 4$ ), in agreement with published data (Stepanov et al., 1997). Indeed, neither ATP compartmentalization nor ATP- $P_i$  exchange should influence the determination of the forward flux parameters by TDST.

### Inversion transfer

An inversion PCr protocol is shown in Fig. 2a as well as the time evolution of  $M_{\gamma\text{ATP}}$  and  $M_{\text{PCr}}$  in a typical heart (Fig. 2b). The mean  $k_f$  and forward CK flux measured by inv-PCr ( $0.55 \pm 0.03 \text{ s}^{-1}$  and  $1.05 \pm 0.06 \text{ MU} \cdot \text{s}^{-1}$  respectively) were similar to those measured by TDST (Table 2). Notice, however, that  $k_{if}$  was higher (and thus  $T_{\text{PCr}}$  was lower) in the inv-PCr protocol. The reverse flux was markedly larger than  $F_f$ , and thus the ratio  $F_f/F_r$ ,  $0.51 \pm 0.04$ , was signifi-

**TABLE 2** Parameters of the forward and reverse CK flux in perfused hearts: comparison of the protocols of time-dependent saturation transfer of  $\gamma$ ATP, inversion transfer of PCr, and inversion transfer of  $\gamma$ ATP analyzed in the two-site two compartment model I

	Saturation $\gamma$ ATP ( $n = 4$ )	Inversion PCr ( $n = 5$ )	Inversion $\gamma$ ATP ( $n = 5$ )
Parameters of the forward CK flux PCr $\rightarrow$ ATP			
$k_f$ ( $s^{-1}$ )	$0.58 \pm 0.04$	$0.55 \pm 0.03$	$1.26 \pm 0.22^*$
$k_{1f}$ ( $s^{-1}$ )	$0.91 \pm 0.05$	$1.01 \pm 0.04$	$1.22 \pm 0.14$
$T_{1PCr}$ (s)	$3.15 \pm 0.28^*$	$2.22 \pm 0.09$	
Flux $F_f$ ( $MU \cdot s^{-1}$ )	$1.02 \pm 0.04$	$1.05 \pm 0.06$	$2.31 \pm 0.38^*$
Parameters of the reverse CK flux ATP $\rightarrow$ PCr			
$k_r$ ( $s^{-1}$ )		$2.16 \pm 0.23$	$1.34 \pm 0.07^*$
$k_{1r}$ ( $s^{-1}$ )		$3.07 \pm 0.19$	$2.97 \pm 0.12$
$T_{1\gamma ATPi}$ (s)		$1.28 \pm 0.29$	$0.62 \pm 0.04$
Flux $F_r$ ( $MU \cdot s^{-1}$ )		$2.15 \pm 0.24$	$1.26 \pm 0.05^*$
$F_f/F_r$		$0.51 \pm 0.04^\dagger$	$1.80 \pm 0.25^{*\dagger}$

All results are mean  $\pm$  SE. PCr and ATP (in magnetization units) were the mean values of control spectra (interpulse delay = 10 s) obtained just before and after the inversion transfer. Flux were expressed in magnetization units  $s^{-1}$ . PCr to ATP ratios were similar in inv-PCr and inv-ATP protocols ( $1.91 \pm 0.10$  and  $1.94 \pm 0.07$ , respectively).

\*Comparison with inv-PCr protocol (significant difference  $p < 0.05$ ).

$^\dagger$ Significantly different from unity ( $p < 0.05$ ).

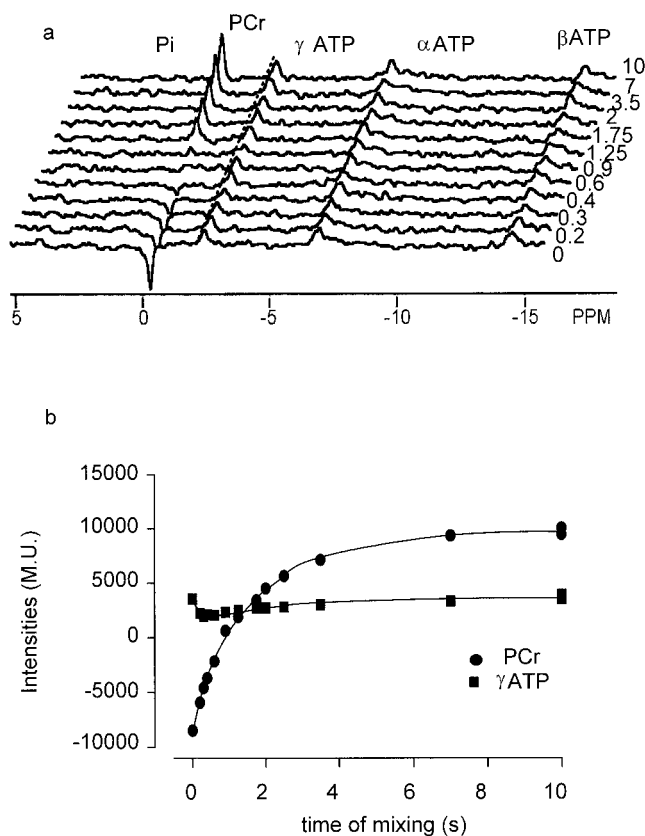
cantly lower than unity ( $p < 0.001$ ). According to our in vitro approach, this suggests that 49% of cellular ATP did not participate in the CK reaction.

Fig. 3 shows a typical inv- $\gamma$ ATP protocol. When analyzed in model I, the mean  $F_f$  appeared to be twice as high as the value determined by TDST or by inv-PCr protocol (Table 2). As a result,  $F_f/F_r$ ,  $1.80 \pm 0.25$ , was significantly different from 1 ( $p < 0.01$ ). Again, by analogy with the in vitro model overestimation of  $F_f/F_r$  could result from the presence of an ATP compartment. Table 3 presents the individual analysis in model I of each heart used in the inv-ATP protocol: the parameters of the reverse flux were determined with small confidence intervals (at most 15% on  $k_r$  and  $k_{1r}$ ). Yet the insufficiency of this model was clearly shown by the impossibility of estimating  $T_{1PCr}$  (in four of five inv- $\gamma$ ATP hearts,  $k_f$  was  $\geq k_{1f}$ ).

Hence, by comparison with the in vitro study, the results of both inv-PCr and inv-ATP protocols could be directly explained by the existence of a fraction of ATP that did not exchange with PCr through CK. However, the importance of this fraction and the impossibility of determining  $T_{1PCr}$  by inv- $\gamma$ ATP suggest that the simple model II was not sufficient to describe myocardial CK flux.

#### Inversion transfer with saturation of $P_i$

Elimination of the effect of an ATP- $P_i$  exchange was achieved by continuous saturation of  $P_i$ . Sat- $P_i$  decreased the steady-state magnetization of both ATP and PCr (by



**FIGURE 2** Inversion of PCr in a representative heart. (a) Stacked plot of the spectra obtained for various times of mixing after the sinc pulse selectively inverting PCr (time of mixing in seconds on the right). The dotted line shows the equilibrium magnetization of the noninverted species expected in the absence of inversion. (b) Variation of PCr and ATP magnetizations (in magnetization units) as a function of the time of mixing.

$15 \pm 1$  and  $14 \pm 1\%$ , respectively,  $n = 10$ ) and affected CK kinetic parameters (Table 4).

In inv-PCr, all parameters of the forward CK flux ( $k_f$  and  $F_f$ ), including  $k_{1f}$  and  $T_{1PCr}$ , were similar, in that protocol, to those measured by saturation of  $\gamma$ ATP (Table 2). Thus TDST and PCr inversion transfer with saturation of  $P_i$  are fully equivalent for the detection of forward flux in myocardium. The ratio  $F_f/F_r$  ( $0.80 \pm 0.06$ ) was still significantly different from 1 ( $p < 0.05$ ). According to our in vitro model, this suggests the existence of a 20% ATP compartmentalization.

In inversion of  $\gamma$ ATP, a complex two-site, three-compartment analysis was required to account for the presence of ATP compartmentalization. Indeed, allowing all parameters to fit without constraints resulted in huge confidence intervals (for instance,  $\sim 200\%$  on  $k_1$  determination). However, the mean values of the forward and reverse parameters obtained (data not shown) were not statistically different from those of Table 4, which presents the analysis performed by imposing the forward flux parameters. The observed ratio  $F_f/F_r$  ( $0.67 \pm 0.11$ ) still suggests the existence

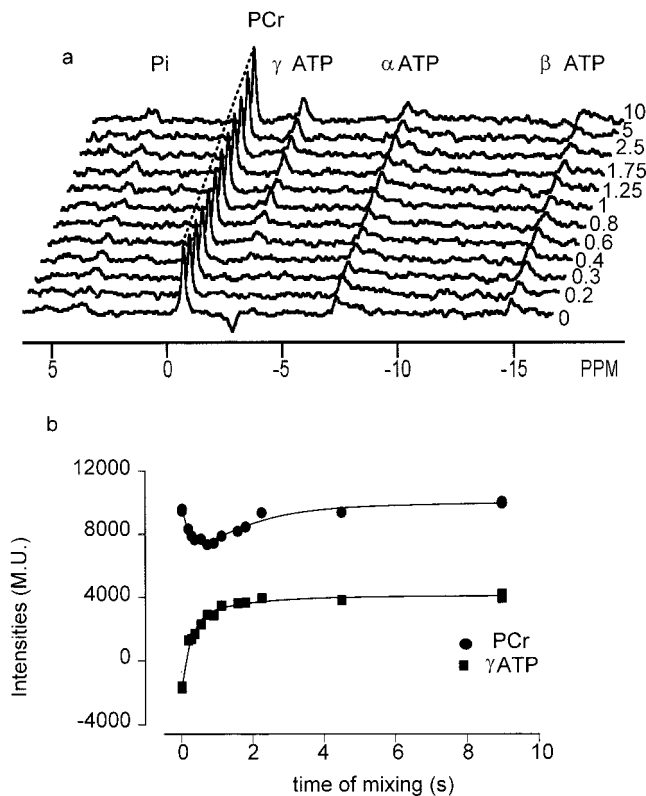


FIGURE 3 Inversion of  $\gamma$ -ATP in a representative heart. Same legend as for Fig. 2. Transfer of magnetization between  $\gamma$ - and  $\beta$ -ATP could not be demonstrated.

of a significant ATP fraction of 33%. Although this triple compartment analysis of inv-ATP was difficult in the heart,  $f_{\text{ATP}}$  was similar to that measured by inv-PCr. No significant difference was found in the apparent intrinsic relaxation time of ATP<sub>2</sub> and ATP<sub>1</sub>;  $T_{1\gamma\text{ATP1}}$  was also similar to that measured by inv-PCr protocol. Notice that with saturation of P<sub>i</sub>, if the presence of the ATP compartment was neglected (i.e., analysis in model I), the mean  $F_r$  value ( $1.01 \pm 0.07 \text{ MU}\cdot\text{s}^{-1}$ ), was similar to  $F_f$  obtained in inv-PCr, as also expected from the in vitro study, suggesting the relevance of the compartmentalization model.

Thus, under conditions supposed to mask the effect of the P<sub>i</sub>  $\rightarrow$  ATP transfer by a continuous saturation of P<sub>i</sub>, the two inversion protocols independently revealed the existence of a significant ATP compartment ( $\sim 20\%$  of myocardial ATP) not involved in the CK reaction.

## DISCUSSION

### Ability of the different techniques to reveal ATP compartmentalization

As shown in vitro, a complete time analysis of the magnetization recoveries in an IT protocol is able to reveal the existence of an ATP compartment isolated from CK. As

expected from the theory, neglecting ATP compartmentalization (i.e., performing the analysis in model I) resulted in an apparent flux discrepancy in both inv-PCr and inv-ATP protocols (an underestimation of  $F_f/F_r$  in the former and its overestimation in the latter; Fig. 1). The same behavior was observed in myocardium (Table 2), clearly confirming the insufficiency of the two-site, two-compartment exchange model to account for myocardial energy transfer.

The possibility of detecting an ATP compartment by NMR depends on the type of NMR transfer protocol (Table 1). For instance, when ATP P<sub>i</sub> exchange was masked, flux discrepancy could not be evidenced by steady state or time-dependent saturation transfer (Ugurbil et al., 1986; Spencer et al., 1988). As discussed in the Theory section and previously reported (Bittl and Ingwall, 1985), such an ATP compartmentalization cannot be revealed by TDST. Furthermore, its effects will be minimized by an analysis of the initial rates of recovery in an IT or a 2D protocol, as easily demonstrated from the Bloch equations (Meyer et al., 1982; Koretsky et al., 1985; DeFuria, 1985). Indeed, no discrepancy between the  $F_f$  and  $F_r$  has ever been revealed by initial rate analysis in muscle (Hsieh and Balaban, 1988; McFarland et al., 1994).

The results of magnetization transfer experiments are also highly dependent on the experimental conditions (Table 1). For example, in glucose substrate and basal working conditions, equal fluxes were observed by a complete analysis of inversion and by steady-state saturation transfer (Degani et al., 1985). The flux discrepancy, when observed, was suggested to be directly related to the level of cardiac performance (Koretsky et al., 1985); indeed both the ATP-P<sub>i</sub> exchange and the amount of ATP present in the mitochondrial matrix (Soboll and Bungler, 1981) are directly related to work. Moreover, most authors, except the group of Ingwall (Bittl and Ingwall, 1985; Bittl et al., 1987; Spencer et al., 1988), observing that the flux discrepancy seen in vivo (Koretsky et al., 1986; Hsieh and Balaban, 1988; Mora et al., 1992) or in hearts using acetate or pyruvate (Nunally and Hollis, 1979; Matthews et al., 1982; Ugurbil et al., 1986; Zweier and Jacobus, 1987) disappeared with glucose utilization (Matthews et al., 1982; Ugurbil et al., 1986; Zweier and Jacobus, 1987), suggested that the contribution of contaminating reactions or ATP compartmentalization was masked by the higher CK flux observed in glucose. Last, various pulse protocols were used in the different studies. Mora et al. (1992), using a long repetition time of 10 s, allowing relaxation of each species, as in our work, could also reveal the apparent flux discrepancy. In contrast, when using a short repetition time of 4 s (similar to  $T_{1\text{PCr}}$ ), like that of Degani et al. (1985), we could no longer find evidence of the flux discrepancy ( $F_f/F_r$  was 1.04 in inv-PCr and 1.08 in inv-ATP,  $n = 2$ ). This might suggest that some reaction contributing to the detected flux was eliminated by fast pulsing. Although the exact contributions of the various factors (work, substrate, pulse conditions) are



**TABLE 3**  $\gamma$ ATP inversion transfer experiments in individual rat hearts: analysis in model I (two-site, two-compartment)

Heart	$k_r$	$k_{1r}$	$T_{1\gamma\text{ATP}}$	$k_f$	$k_{1f}$	$T_{1\text{PCr}}$
1	1.58 (0.18)	3.38 (0.31)	0.56	2.08 (0.51)	1.79 (0.29)	-3.45
2	1.36 (0.16)	3.11 (0.32)	0.57	0.99 (0.34)	0.79 (0.17)	-5.00
3	1.14 (0.10)	3.02 (0.21)	0.53	1.51 (0.33)	1.18 (0.16)	-3.03
4	1.22 (0.11)	2.68 (0.21)	0.68	1.11 (0.31)	1.15 (0.17)	
5	1.38 (0.12)	2.68 (0.20)	0.77	0.62 (0.27)	1.17 (0.17)	1.82
Mean	1.34	2.97	0.62	1.26	1.22	-2.42
SE	0.07	0.12	0.04	0.22	0.14	1.28

Parameters are defined in the Theory section. The interval of confidence of the fit is shown in parentheses for each parameter. Variability between hearts is shown by the mean  $\pm$  SE values. Notice that this analysis allowed the determination of  $T_{1\gamma\text{ATP}}$  but not of  $T_{1\text{PCr}}$  (in four of five hearts  $k_f$  is equal or superior to  $k_{1f}$ ).

difficult to appreciate, we believe that they may explain the impossibility of experimentally detecting the presence of an ATP compartment in some previous work.

We further demonstrated that when  $P_i$  magnetization was continuously saturated during inversion experiments, the parameters of forward CK flux were similar in protocols of inversion (Table 4) and saturation of  $\gamma$ ATP (Table 2) and thus can be consistently determined. Absolute flux values (Table 5) were also similar to  $F_f$  previously measured by  $\gamma$ ATP saturation (Koretsky et al., 1986; Ugurbil et al., 1986; Stepanov et al., 1997), in which neither ATP compartmentation nor ATP- $P_i$  exchange induces errors in CK parameters. Although Koretsky et al. (1985) and Brindle (1988) previously suggested that the inversion and the saturation protocols differ in their capacity for CK flux detection, here

we show that, when inversion was performed with saturation of  $P_i$ , the two techniques were equivalent for the detection of the forward flux. The advantage of the complete analysis of an IT experiment over the other experimental protocols is, however, its ability to quantify ATP compartmentalization by two independent methods (inv-PCr and inv- $\gamma$ ATP).

### Flux discrepancy observed with saturation of $P_i$ can be understood in the hypothesis of an ATP compartmentalization

The origin of the observed discrepancy of forward and reverse flux has been alternately attributed to the existence

**TABLE 4** Comparison of the parameters of forward and reverse CK fluxes measured in the perfused heart by inversion PCr and inversion of  $\gamma$ ATP under continuous saturation of  $P_i$  magnetization

	Inversion PCr ( $n = 5$ )	Inversion $\gamma$ ATP ( $n = 5$ )	
		Analysis in model I	Analysis in model II
Parameters of the forward CK flux			
$k_f$ ( $s^{-1}$ )	$0.56 \pm 0.03$	$0.78 \pm 0.05^*$	$0.57 \pm 0.03$
$k_{1f}$ ( $s^{-1}$ )	$0.88 \pm 0.03$	$1.00 \pm 0.08$	$0.88 \pm 0.03$
$T_{1\text{PCr}}$ (s)	$3.22 \pm 0.16$	$5.4 \pm 1.1$	3.22 (fixed)
Flux $F_f$ ( $\text{MU} \cdot s^{-1}$ )	$0.96 \pm 0.12$	$1.32 \pm 0.10$	0.96 (fixed)
Parameters of the reverse CK flux			
$k_r$ ( $s^{-1}$ )	$1.36 \pm 0.16$	$1.19 \pm 0.08$	$1.85 \pm 0.17^*$
$k_{1r}$ ( $s^{-1}$ )	$3.39 \pm 0.30$	$2.94 \pm 0.12$	$3.42 \pm 0.33$
$T_{1\gamma\text{ATP1}}$ (s)	$0.53 \pm 0.06$	$0.55 \pm 0.02$	$0.70 \pm 0.09$
Flux $F_r$ ( $\text{MU} \cdot s^{-1}$ )	$1.22 \pm 0.16$	$1.01 \pm 0.07$	$1.63 \pm 0.23$
Analysis			
$F_f/F_r$	$0.80 \pm 0.06^\dagger$	$1.32 \pm 0.06^{*\dagger}$	$0.67 \pm 0.11^\dagger$
$f_{\text{ATP}}$ (%)	$20 \pm 6$		$33 \pm 11$
$T_{1\gamma\text{ATP2}}$ (s)			$0.66 \pm 0.06$

Continuous saturation of  $P_i$  decreased the steady-state magnetization of PCr and ATP. This decrease, expressed as a percentage of steady-state magnetization  $M^s$  (measured in the absence of  $P_i$  saturation with a 10-s repetition time), was  $14 \pm 1\%$  for PCr and  $15 \pm 1\%$  for  $\gamma$ ATP ( $n = 10$ ). The PCr to ATP ratio was similar in the two protocols ( $1.87 \pm 0.11$  and  $2.01 \pm 0.14$  in inv-PCr and inv-ATP, respectively). The analysis in model II was performed with fixed parameters to improve the interval of confidence of the fit.  $T_{1\text{PCr}}$  was measured in both protocols of inv-PCr with  $P_i$  saturation and TDST. Because inv-PCr and inv- $\gamma$ ATP were performed on different hearts,  $F_r$  (and not  $k_r$ ) was fixed to account for any eventual variation in PCr content of the individual hearts.

\*Comparison with inv-PCr protocol (significant difference  $p < 0.05$ ).

$^\dagger F_f/F_r$  significantly different from unity ( $p < 0.05$ ) (concomitantly  $f_{\text{ATP}}$  differed from zero) supporting the hypothesis of ATP compartmentalization.

**TABLE 5 Summary of flux measurements in myocardium**

Type of protocol	<i>n</i>	$F_f$ (mM·s <sup>-1</sup> )	$F_r$ (mM·s <sup>-1</sup> )
Time-dependent saturation of $\gamma$ ATP	4	7.3 ± 0.3	
Inversion of PCr	5	7.5 ± 0.4	15.4 ± 1.7*†
Inversion of $\gamma$ ATP	5	16.5 ± 2.7*	9.0 ± 0.4†
Inversion of PCr with saturation of $P_i$	5	6.9 ± 0.8	8.7 ± 1.1‡
Inversion of $\gamma$ ATP with saturation of $P_i$	5	9.5 ± 0.8‡	7.2 ± 0.5‡

\*Significant difference between fluxes measured by inversion protocols and  $F_f$  measured by TDST ( $p < 0.05$ , variance analysis and Student-Newman-Keuls).

†Significant difference between forward and reverse flux in each protocol ( $p < 0.05$ , paired *t*-test).

‡Influence of  $P_i$  saturation, significant difference in the presence and absence of  $P_i$  saturation ( $p < 0.05$ , *t*-test).

of an ATP compartmentation (model II; Nunnally and Hollis, 1979), the exchange of ATP with species other than PCr (model III; Ugurbil et al., 1986; Spencer et al., 1988), or the influence of a small invisible ATP pool in saturation protocols (Koretsky et al., 1985; Zahler et al., 1987). We do not favor the last as the source of flux discrepancy observed in our inversion protocol. Indeed, in a saturation protocol, the influence of a small invisible ATP pool on the evolution of  $M_{PCr}$  could be detected. However, in a pulse labeling technique like ATP inversion, the change in PCr magnetization being proportional to the size of the inverted compartment, such a small invisible ATP pool would have a minimal effect on the exchange, as discussed earlier (Koretsky et al., 1985; Brindle, 1988). Furthermore, this invisible ATP must be small because the ATP content of a normoxic heart is identical when measured by NMR or biochemistry (Humphrey and Garlick, 1991). Thus the exclusive hypothesis of an invisible ATP pool would not be able to account for our inversion data.

The continuous saturation of  $P_i$  resonance makes it possible to mask the influence of the ATP- $P_i$  exchange on the measured CK kinetics (Ugurbil et al., 1986). We indeed observed a clear convergence between the data obtained in vitro and those obtained in perfused rat heart with sat- $P_i$ . All data of both inv-ATP and inv-PCr protocols (Table 4) could thus be understood in the hypothesis of an ATP compartment that does not exchange with PCr in model II. This two-site, three-compartment model is a minimal model for cardiac CK explored with saturation of  $P_i$ , i.e., a model fitting the data with the smallest number of unknown parameters (two fluxes, one fraction of ATP and  $T_1$ s).

### How to understand the data obtained without saturation of $P_i$

Sat- $P_i$  decreased the steady-state magnetization of both PCr and ATP by ~15%, as already observed (Ugurbil et al., 1986). Furthermore, sat- $P_i$  affected kinetic parameters of the forward and reverse CK, as shown by comparison of Tables 4 and 2. Considering the only consequence of sat- $P_i$  to be the masking of  $P_i \rightarrow$  ATP exchange, it should be possible to theoretically reconstruct the data of Table 2 simply by adding the influence of this exchange to the experimental

results of Table 4. The simulation (shown in the Appendix and Table 6) suggests that experimental data of Table 2 and the decrease in steady-state magnetization induced by  $P_i$  saturation were not fully described by the models considered. As already suggested in the analysis of saturation transfer protocol (Brindle, 1988), the mask of  $P_i \rightarrow$  ATP exchange per se is not expected to have such an influence on CK flux determination. Moreover, the equivalent decrease in steady-state magnetizations  $M_{PCr}^\infty$  and  $M_{\gamma ATP}^\infty$  induced by sat- $P_i$  was not anticipated (Table 6). This suggests that sat- $P_i$  might not just eliminate the contribution of the ATP- $P_i$  exchange, but might also affect other cellular reactions or metabolites implying PCr: an exchange between PCr and ATP<sub>2</sub> appears to be a good candidate for contributing to the decreased  $M_{PCr}^\infty$ . In conclusion, model IV should be refined to account for the results observed in the absence of  $P_i$  saturation.

### Physiological considerations and their expected NMR consequences

The model developed here allowed us to explain both inversion experiments with sat- $P_i$ , suggesting that the system studied by NMR could be mimicked with the minimal simple assumption of an ATP not being involved in the CK reaction. Moreover, this fraction, 20–33% of total cellular ATP, was in the range of mitochondrial ATP content described in the literature for isolated cardiac mitochondria, isolated cardiac myocytes, or hearts performing medium work (Asimakis and Sordall, 1981; Soboll and Bungler, 1981; Geisbuhler et al., 1984). In the hypothesis of ATP<sub>2</sub> localized in the mitochondrial matrix (ATP<sub>2</sub> = ATP<sub>m</sub>), a more physiological scheme can be proposed. Indeed, we have not considered until now the NMR complexity arising from the specific localization of CK isoforms in myocardium (Nunnally and Hollis, 1979; Koretsky et al., 1985; Zahler et al., 1987), particularly the high proportion of mitochondrial CK (mito-CK  $\cong$  20% of total CK) localized in the intermembrane space of mitochondria in the vicinity of translocase. Fig. 4, which schematizes the myocardial phosphorus exchanges, includes cytosolic and mitochondrial CK and the restriction of diffusion for CK metabolites, which separates their function.

**TABLE 6 Comparison of the change observed with saturation of  $P_i$  and the simulation of the effect of the ATP- $P_i$  exchange**

	$F_d/F_r$		Decrease in steady-state magnetization induced by sat- $P_i$	
	Inv-PCr prot.	Inv-ATP prot.	ATP	PCr
Experimental data: effect of sat $_i$				
With sat- $P_i$	$0.80 \pm 0.06$	$1.32 \pm 0.06$	$15 \pm 1$	$14 \pm 1$
Without sat- $P_i$	$0.51 \pm 0.04$	$1.80 \pm 0.25$		
Computed data: simulation of the influence of the ATP- $P_i$ exchange				
Model III	0.94	1.25	13	8
Model IV	0.79	1.74	26	11
Model IVb	0.81	1.17	19	6

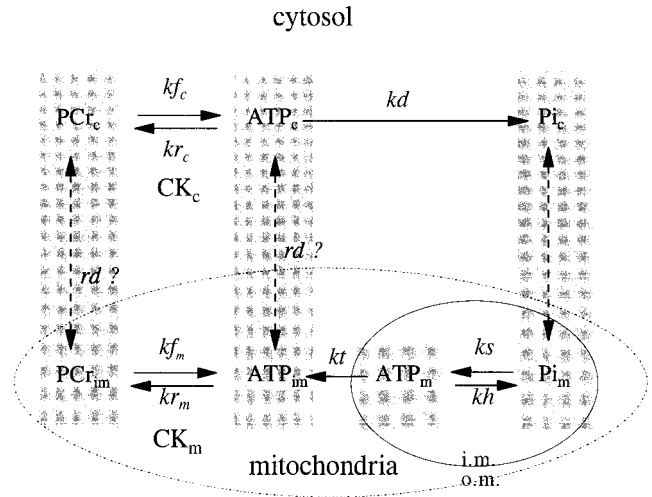
Experimental data: with sat- $P_i$  (data of Table 4), without sat  $P_i$  (data of Table 2) (for  $F_d/F_r$   $n = 5$ ; for the decrease in steady-state magnetization,  $n = 10$ ).

Decrease in steady-state magnetization induced by sat- $P_i$ : for ATP =  $(M_{ATP}^{\infty} - M_{ATP}^*)/M_{ATP}^{\infty}$  and for PCr =  $(M_{PCr}^{\infty} - M_{PCr}^*)/M_{PCr}^{\infty}$  with  $M^{\infty}$  and  $M^*$  = steady-state magnetization in the absence or presence of  $P_i$  saturation, respectively.

By these hypotheses the time variation of  $ATP_c$  under sat- $P_i$  is described by Eq. 4 and that of  $ATP_m$  by  $dM_{ATP_m}/dt = -(k_t + k_h + 1/T_{1\gamma ATP_m})(M_{\gamma ATP_m} - M_{\gamma ATP_m}^*)$ . Because  $ATP_m$  does not participate in the CK reaction, inv-PCr does not affect this pool. In case of inv-ATP, the return to equilibrium of the  $ATP_m$  pool is independent of CK. Thus in terms of NMR,  $ATP_m$  will also behave as if it were isolated from the CK reaction, despite the fact that it is in physiological exchange with  $ATP_c$  (as shown in Fig. 4). Hence there is no contradiction between isolation of  $ATP_m$  by NMR procedure and the fact that up to 90% of cellular ATP is ultimately labeled in a skeletal muscle when net phosphoryl transfer flux is measured by  $^{18}O$  exchange kinetics (Zelevnikar and Goldberg, 1991).

The mitochondrial ATP synthesis and its hydrolysis by myofibrillar, sarcoplasmic reticulum, and sarcolemmal ATPases imply, when translated to an NMR experiment, that sat- $P_i$  would mainly affect mitochondrial metabolites when glycolytic ATP production is negligible (which is the case here because of acetate utilization). Thus mito-CK could be an indirect target of sat- $P_i$  (Fig. 4). In this hypothesis, considering an exchange of  $ATP_2 \rightarrow PCr$  through mito-CK flux and metabolite steady state, an equal decrease in  $M_{PCr}^{\infty}$  and  $M_{ATP}^{\infty}$  could be simulated when the formalism of the Appendix is used. Notice that our results do not exclude the possible existence, however, between  $ATP_m$  and PCr, of a small intermediate ATP pool ( $ATP_{im}$ ) exchanging with CK, which might be the invisible ATP pool previously suggested (Koretsky et al., 1985; Zahler et al., 1987).

Finally, the comparison of data obtained with and without sat- $P_i$  might make it possible to quantify the contribution of



**FIGURE 4** Schematic representation of myocardial CK. The model takes into account compartmentalization of mitochondrial CK isoenzyme ( $CK_m$ ) located in the intermembrane in the vicinity of translocase.  $CK_c$  refers to both cytosolic and myofibrillar CK. Gray areas, metabolite compartment:  $P_i$ ,  $ATP_1$ ,  $ATP_2$  and PCr. Subscript m relates to mitochondrial matrix, c to cytosol, and im to intermembrane space (between the inner and outer mitochondrial membranes).  $k$ , rate constants:  $k_f$  and  $k_r$ , CK flux of ATP and PCr synthesis, respectively.  $k_t$ , rate constant of ATP efflux from mitochondrial matrix by adenine nucleotide translocase. The influx of ATP to the matrix was neglected because under high activation of mitochondrial respiration (i.e., when the heart works) the translocase predominantly extrudes ATP from the matrix to the mitochondrial intermembrane space.  $k_s$  and  $k_h$ , rate constants of mitochondrial ATP synthesis and hydrolysis, respectively. The presence of oxidative substrate (acetate or pyruvate) is well known to inhibit myocardial glycolytic ATP production in normoxia. Thus the  $P_i \rightarrow ATP$  reaction can be considered in our experimental design to be restricted to the mitochondrial matrix.  $k_d$ , rate of ATP hydrolysis, referring to the cytosolic ATP- $P_i$  flux. The myofibrillar ATPase, responsible for 90% of  $ATP \rightarrow P_i$  hydrolysis in the myocardium, is irreversible (thus the reverse cytosolic flux  $k_d$ , was neglected).  $rd?$ , putative restriction of diffusion. ATP exchange with species other than  $P_i$  was considered negligible because a transfer of magnetization from  $\gamma ATP$  to  $\beta ATP$ , indicative of a significant adenylate kinase activity, was never observed.

mito-CK flux in the whole organ. This hypothesis is currently being tested under various physiological conditions. This also suggests the necessity of considering not only cytosolic CK but also the function of bound enzymes and restrictions of diffusion to fully understand the NMR data. Indeed, the activities of mitochondrial and cytosolic CK (Aliev and Saks, 1997), as well as the consequences of mito-CK activity on flux determined by saturation transfer (Zahler et al., 1987), have already been modeled but still await extensive experimental validation. We believe that our approach of IT analysis has the potential to produce more experimental information on subcellular exchanges.

**APPENDIX**

Our minimal model of ATP compartmentalization (model II) was fully compatible with the results of the experiments with the saturation of  $P_i$

(Table 4). Thus if saturation of  $P_i$  simply masks the  $P_i$ -ATP exchange, it must be theoretically possible, knowing the flux of ATP synthesis, to mimic the results of the inversion protocol without saturation of  $P_i$  (Table 2).

The rationale was to generate computed data, using the CK parameters obtained with saturation of  $P_i$  (Table 4), and to introduce an ATP- $P_i$  exchange estimated from cardiac work. Computed data were then analyzed in a two-site, two-compartment exchange system (model I). Simulation produced evidence of the errors in the  $F_i/F_r$  ratio induced by the omission of both ATP- $P_i$  fluxes and the ATP compartment and can be compared with the experimental data of Table 2. Furthermore, we computed the decrease in steady-state magnetization of  $\gamma$ ATP and PCr expected in each model from a continuous saturation of  $P_i$ .

Bloch equations in model IV are described by

$$\frac{d}{dt} \begin{pmatrix} M_{\text{PCr}} \\ M_{\gamma\text{ATP1}} \\ M_{\gamma\text{ATP2}} \\ M_{P_i} \end{pmatrix} = \begin{pmatrix} -k_{1f} & k_r & 0 & 0 \\ k_f & -k_{1r} & 0 & k_d' \\ 0 & 0 & -k_{1h} & k_s \\ 0 & k_d & k_h & -k_{1s} \end{pmatrix} \cdot \begin{pmatrix} M_{\text{PCr}} - M_{\text{PCr}}^\infty \\ M_{\gamma\text{ATP1}} - M_{\gamma\text{ATP1}}^\infty \\ M_{\gamma\text{ATP2}} - M_{\gamma\text{ATP2}}^\infty \\ M_{P_i} - M_{P_i}^\infty \end{pmatrix}$$

where

$$k_{1f} = k_f + \frac{1}{T_{1\text{PCr}}}$$

$$k_{1r} = k_r + k_d + \frac{1}{T_{1\gamma\text{ATP}}}$$

$$k_{1h} = k_h + \frac{1}{T_{1\gamma\text{ATP}}}$$

$$k_{1s} = k_s + k_d' + \frac{1}{T_{1P_i}}$$

## Input parameters

The ATP synthesis rate ( $2.3 \text{ mM} \cdot \text{s}^{-1}$ ) was inferred from the work and oxygen consumption ( $10 \mu\text{mol}/\text{O}_2/\text{min}/\text{gWW}$ ) developed, assuming a P/O ratio of 3, as previously described (Stepanov et al., 1997). The ATP synthesis rate was thus threefold lower than CK flux, in agreement with previous data (Matthews et al., 1982; Koretsky et al., 1986; Stepanov et al., 1997).  $T_{1P_i}$  was taken to be equal to 1 s (from Brindle, 1988). Both  $T_{1\gamma\text{ATP1}}$  and  $T_{1\gamma\text{ATP2}}$  (considered equals) were calculated from the relation  $1/T_{1\gamma\text{ATP}}^* = k_d + 1/T_{1\gamma\text{ATP}}$  (see Theory), assuming  $k_d\text{ATP}_1^\infty$  flux (net utilization) = 1/3 of CK flux:  $T_{1\gamma\text{ATP1}} = T_{1\gamma\text{ATP2}} = 0.7 \text{ s}$ . The fraction of  $f_{\text{ATP}}$  was 0.2 (from Table 4).

The results of the simulation are shown in Table 6 for model IV and for an extension of model IVb that considers the possibility of an additional exchange  $\text{ATP}_2 \rightarrow \text{ATP}_1$  (and unidirectional  $\text{ATP}_1 \rightarrow P_i$  and  $P_i \rightarrow \text{ATP}_2$  fluxes to ensure the steady state of metabolites). To compare to the influence of ATP- $P_i$  exchange in a saturation transfer protocol saturation (Brindle, 1988), we also used model III (although this model was shown to be incompatible with our experimental data with saturation of  $P_i$ ).

Simulation of the effect of ATP  $P_i$  exchange in model IV could mimic the experimental change in  $F_i/F_r$  induced by sat- $P_i$  in inv-ATP but not those in inv-PCr (from 0.80 to 0.51); in fact the decrease in  $F_i/F_r$  observed in inv-PCr under sat- $P_i$  was expected in none of the models. Furthermore, the equal decline in steady-state magnetization of PCr and ATP observed under sat- $P_i$  could not be simulated; in all models the decrease in  $M_{\text{ATP}}^\infty$  is expected to be higher than that of  $M_{\text{PCr}}^\infty$ . In conclusion, none of the models

tested appeared to be adequate to mimic the totality of the observed effects of sat- $P_i$  (i.e., the results of inv-PCr and of inv-ATP of Table 2 and the steady-state decrease in PCr and ATP magnetization induced by saturation of  $P_i$ ).

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