Creatine kinase-catalyzed ATP-phosphocreatine exchange:

Comparison of ^{31}P-NMR saturation transfer technique and radioisotope tracer methods

V.V. Kupriyanov, N.V. Lyulina, A.Ya. Steinschneider, M.Yu. Zueva and V.A. Saks

Institute of Experimental Cardiology, USSR Cardiology Research Center of the Academy of Medical Sciences, Moscow, USSR

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Unidirectional fluxes from ATP to phosphocreatine, catalyzed by the MM isoenzyme of creatine kinase, were measured by both the ^{31}P-NMR saturation transfer technique and radioisotope tracer ([γ-^{32}P]ATP) method. It was found that at 30–37°C and pH 7.4, over a wide range of [phosphocreatine]/[creatine] (from 0.2 to 5.0) ratios, both methods gave the same results, showing that magnetization transfer allows determination of real fluxes under 'physiological' conditions. However, at [PCr]/[Cr] ratios higher than 5 ($[ADP]_{free} < 30\ \mu M$) or at lower temperatures ($t < 15$°C, [PCr]/[Cr] ~ 1), the fluxes assessed by saturation transfer were somewhat faster than those detected by the radioisotope tracer method. These data imply that under physiological conditions phosphoryl group transfer is actually the rate-determining step of the creatine kinase reaction. In contrast, at high [PCr]/[Cr] ratios or at lower temperatures, control may be shifted from phosphoryl group transfer or distributed among other steps of the reaction.

Creatine kinase *ATP-phosphocreatine exchange* *^{31}P-NMR* *Saturation transfer* *Unidirectional flux* *Temperature dependence*

1. INTRODUCTION

NMR magnetization transfer is now widely used for measurements of enzyme-catalyzed fluxes in vitro and in living tissues [1–7]. However, there are several problems concerning the reliability of this technique for these purposes which have been discussed in detail earlier [8,9]. The most straightforward way to answer this question is by means of direct comparison of enzyme-catalyzed fluxes in vitro measured by NMR magnetization transfer with those measured independently by radioisotope tracer methods. A somewhat similar approach has been applied to the creatine kinase reaction where the rates of γ-P(ATP) $\longleftrightarrow$ PCr exchange assessed by saturation transfer were compared with those of phosphocreatine $\longleftrightarrow$ creatine exchange detected with ^{15}N-labeled creatine [10]. However, the rates of these two exchanges are not necessarily equal, since they involve different reaction steps (scheme 1). For this reason magnetization transfer between γ-P(ATP) and phosphocreatine should be compared with radioisotopic [γ-^{32}P]ATP $\longleftrightarrow$ phosphocreatine exchange.

It has been found in this work that the γ-phosphoryl group exchange rates catalyzed by isolated MM creatine kinase determined by both ^{31}P-NMR saturation transfer and radioactive ^{32}P tracer methods are similar at different [phosphocreatine]/[creatine] ratios at 30–37°C. This demonstrates the reliability of saturation transfer data and provides evidence in support of the idea that phosphoryl group transfer is the rate-determining step in the creatine kinase reaction under physiological conditions.

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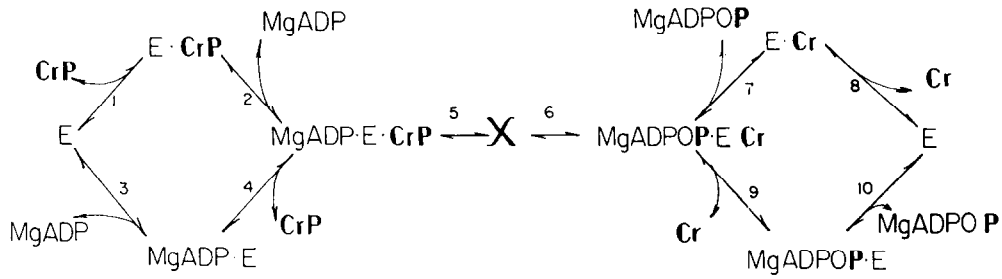

Scheme 1.

2. MATERIALS AND METHODS

2.1. *Measurements of PCr ⟷ ATP fluxes by ^{31}P-NMR saturation transfer*

Measurements were carried out using a WM-500 NMR spectrometer (Bruker, FRG) operating at the standard frequency of 202.45 MHz for ^{31}P nuclei, as described [7,11]. Selective saturation of desired signals was performed by the 'Dante' method [12]. Typical parameters of the Dante sequence were as follows: pulse duration, 1–1.5 μs; interpulse delay, 100–200 μs; number of pulses in a sequence of minimal duration (0.3 s), 1500–3000. Longer saturation times (0.6–15 s) were obtained by applying an integral number (from 2 to 50) of sequences of minimal duration separated by 300 μs delays. The saturating sequence was immediately (1 ms) followed by a 90° sampling pulse (40–56 μs). The relaxation delay was 10–15 s, the number of scans, 16–32, and the memory size, 8–16 K.

The incubation medium contained 20–30 mM Hepes-Na (pH 7.4), 110 mM K acetate, 30 mM KCl, 10 mM Mg acetate, 8 mM ATP, [PCr] + [Cr] = 30 mM, 0.5 mM dithiothreitol, 0.5 mM EGTA-Na, 4–30 mg/ml MM creatine kinase from rat skeletal muscle and 20% D_2O for field-frequency lock.

2.2. *Measurements of creatine kinase fluxes by radioisotope exchange: [γ-^{32}P]ATP ⟷ PCr*

This exchange was assayed in the same medium as saturation transfer, using 0.4 mg/ml (~80 IU/ml) creatine kinase. The incubation mixture was preheated for 15 min at 37°C and then equilibrated at the temperature required (up to 2 h at 0°C) to reach thermodynamic equilibrium. The exchange reaction was then initiated by the introduction of a small volume of [γ-^{32}P]ATP as tracer. The reaction was terminated by adding an equal volume of ice-cold 6% perchloric acid to the incubation mixture. After removal of protein, the acidic extract was diluted 2 times with 6% perchloric acid containing 100 mM PCr and 100 mg/ml charcoal (Norit A) to absorb ATP [13]. Such treatment at 0°C removed about 97% of the ATP and decreased the phosphocreatine content by no more than 10%. Radioactivity of these samples after separation of charcoal was counted in water on a Rack Beta 1215 scintillation counter (LKB, Sweden). Rate constants were calculated from either initial slopes of curves of radioactivity incorporated into phosphocreatine or plotting $\ln(CPM_\infty - CPM_t)$ vs time, where CPM_t and CPM_∞ represent the current radioactivity of PCr at time t and the equilibrium radioactivity at infinity, respectively. The slopes of the straight lines on semilogarithmic coordinates allow one to determine $(k_1 + k_2)$, which is defined at equilibrium as $k_1[ATP]_{eq} = k_2[PCr]_{eq}$ = flux and therefore k_2 = slope/(1 + [PCr]/[Cr]). Both approaches gave similar results.

2.3. *Kinetic measurements*

The initial rates of the creatine kinase reaction in both directions were determined spectrophotometrically at 340 nm using coupled enzyme assays: glucose-6-phosphate dehydrogenase/hexokinase (reverse reaction) and pyruvate kinase/lactate dehydrogenase (forward reaction) [14]. The concentrations of substrate pairs were varied and the K_m values and maximal rates (V_m) were determined from analysis of double-reciprocal plots.

2.4. *Reagents*

Creatine, nucleotides, dithiothreitol, EGTA and Hepes were purchased from Sigma (USA). Phosphocreatine (disodium salt) was obtained from Skiapparelli Farmaceutici (Italy). [γ-^{32}P]ATP was supplied by Amersham (England). The MM isoenzyme of creatine kinase was isolated from rat skeletal muscle according to Keutel et al. [15] as described earlier [7].

3. RESULTS AND DISCUSSION

Fig.1 shows the dependence of the creatine kinase-catalyzed unidirectional flux (γ-P(MgATP) $\longrightarrow$ PCr) on the phosphocreatine/creatine ratio in vitro for a fixed pool of total creatine ([PCr] + [Cr] = 30 mM) under conditions mimicking physiological: temperature, 37°C; pH 7.4; [ATP] + [ADP] = 8 mM; free Mg^{2+}, approx. 2 mM, 140 mM K^+; ionic strength, approx. 350 mM. This dependence was obtained by two methods, firstly ^{31}P-NMR saturation transfer [5] and, secondly, the radioisotope tracer method using γ-^{32}P-labelled ATP (for details see section 2). We can see the closeness of these two sets of data over a wide range; both fit well the theoretical bell-shaped curve calculated on the basis of the kinetic equation for the creatine kinase reaction and known values of kinetic constants for these experimental conditions [4]. Only at high [PCr]/[Cr] ratios (>5) and consequently at high [ATP]/[ADP] are saturation transfer-detected fluxes somewhat higher than the radioisotope and theoretically calculated fluxes. An interesting peculiarity of this dependence is the remarkable stability of the flux over a broad range of [PCr]/[Cr] ratios. Thus, an increase in this ratio from 0.2 to 5 (25-times) results in approx. 30% changes of flux with respect to the maximum. These data demonstrate, firstly, that creatine kinase-catalyzed fluxes determined by saturation transfer really correspond to the true fluxes measured on the basis of [γ-^{32}P]ATP $\longrightarrow$ PCr exchange under physiological conditions. Therefore, we may suppose that magnetization

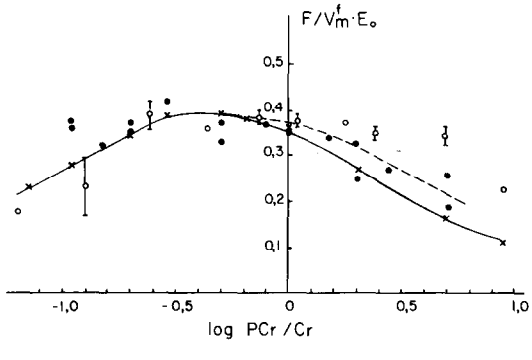

Fig.1. Dependence of creatine kinase-catalyzed fluxes PCr $\rightleftharpoons$ γ-P(ATP) on the [PCr]/[Cr] ratio. (○) NMR-detected fluxes, (●) fluxes determined with tracer ([γ-^{32}P]ATP); (×) calculated fluxes. Details are given in section 2. Values of fluxes are normalized to the enzyme concentration and maximal rate of the forward reaction at pH 7.4 and 37°C since experiments were done with different batches of the enzyme with different specific activity.

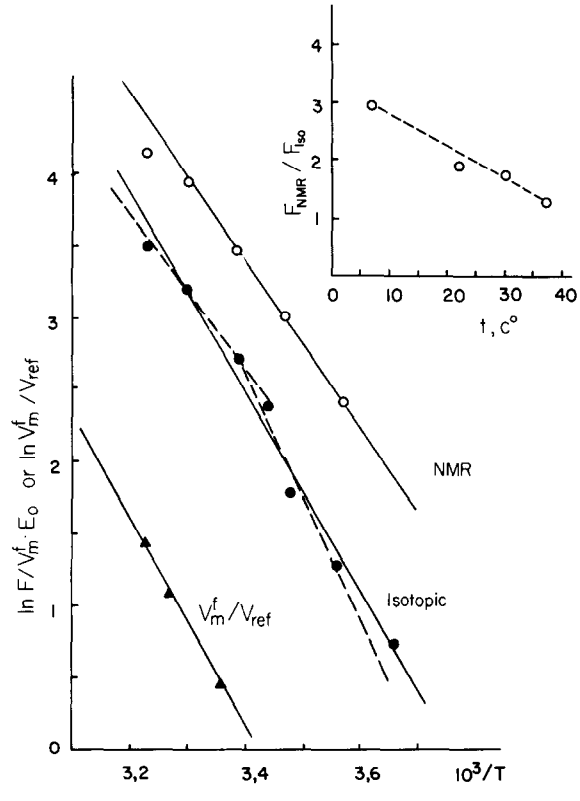

Fig.2. Temperature dependence of PCr $\rightleftharpoons$ ATP exchange rates catalyzed by creatine kinase and maximal rate of the reverse reaction. V_m is referred to enzyme activity determined under the standard conditions which served as a measure of active enzyme content. For other details see section 2. Inset: dependence of the ratio of fluxes determined by NMR and radioisotope methods (F_{NMR}/F_{Iso}) on temperature.

transfer provides correct information on the creatine kinase reaction rates in vitro and probably, in living systems under appropriate circumstances. Secondly, these data support the early findings of Morrison and Cleland [16] and our recent conclusions based on equality of the rates of PCr ⟷ ATP and ATP ⟷ ADP exchange assessed by saturation transfer [8] that phosphoryl group transfer is the rate-determining step of the creatine kinase reaction at 30–37°C. In fact, the overall flux between PCr and ATP, determined using radioisotope tracer (γ-^{32}P of ATP), in general depends on all elementary reaction rate constants, including the dissociation and association of PCr and ATP (scheme 1). In contrast, the flux measured by ^{31}P-NMR saturation transfer is mostly determined by the phosphoryl group transfer step because of the closeness of the chemical shifts of the ^{31}P signal of free and enzyme-bound phosphocreatine as well as that of the γ-^{31}P signal of free and bound MgATP as was recently shown in our papers [8,9]. Therefore, if phosphoryl group transfer was not the rate-limiting, the real flux could be lower than that detected by magnetization transfer and consequently the latter would be overestimated.

However, a tendency to this phenomenon can be noticed at high [PCr]/[Cr] ratios (>5), when the free MgADP concentration is lower than 20 μM, i.e. equal to or lower than the enzyme concentration (10–100 μM). Therefore, we can assume that steps of dissociation and/or association of MgADP become more pronounced in the control of overall flux through the enzyme at very low ADP concentrations.

Since, in general, the temperature coefficients for different steps of enzyme reaction can be different, the position of the rate-limiting step can be shifted with variation in temperature. Therefore, we have investigated the temperature dependence of creatine kinase fluxes from ATP to PCr determined by saturation transfer and radioisotope tracer methods and made a comparison of such a dependence for maximal rates of this reaction in both directions found from steady-state kinetics. The results of this study are shown in fig.2. It is evident that all three straight lines in the Arrhenius plot are almost parallel and the apparent activation energy calculated from their slopes is between 12 and 15 kcal/mol. This value is identical to those found previously with MM creatine kinase from rabbit skeletal muscle [17].

Note here that fluxes under equilibrium conditions were measured at the following concentrations of substrates: [PCr] = 15 mM, [Cr] = 15 mM, [ATP] ≃ 8 mM and [ADP] ≃ 80 μM or lower depending on temperature. The concentrations of creatine and especially of ADP are not saturating, since their respective K_m values are 10 and 0.2 mM at 30–37°C. Therefore, the temperature dependences of these K_m values could contribute to the observed dependence of flux together with those for V_m values. However, the closeness of the apparent activation parameters for V_m and radioisotope flux shows that the contribution of K_m in the temperature dependence of this flux is negligible, probably because of a weak dependence of these values on temperature in the range investigated. This conclusion is correct if phosphoryl group transfer is the rate-determining step of the overall reaction throughout the temperature range studied. If this is not the case, the control step can be shifted or distributed with decreasing temperature and therefore V_m cannot be exclusively referred to the phosphoryl group transfer step. From this viewpoint a difference in the apparent activation energies (approx. 3 kcal/mol) of fluxes determined by magnetization transfer (~12 kcal/ml) and radioisotope tracer methods (~15 kcal/mol) can be ascribed to some shift of the control step from phosphoryl group transfer. In fact, as pointed out above, magnetization transfer depends only on the phosphoryl group transfer step [8,9] and therefore shift or distribution of control to other step(s) resulting in a higher apparent activation energy of the overall exchange does not affect magnetization transfer. In contrast, radioisotopic exchange is sensitive to these changes in position of the controlling step(s). In fact, the ratio of flux determined by saturation transfer to that measured using radioisotope tracer rose from 1.25 to 3 as the temperature fell from 37 to 7°C, as shown in the inset to fig.2. Moreover, careful inspection of the Arrhenius plot for isotopic exchange shows some break of this line at 25°C and its two linear parts are characterized by apparent activation energies around 12 (upper) and 18 kcal/ml (lower). This phenomenon can be explained by the appearance of another rate-controlling step due to the decrease in temperature;

the contribution of this step is displayed as the increase in apparent activation energy of the overall reaction. This conclusion does not contradict the data of Rao and Cohn [18], demonstrating that at low temperature (4–7°C) phosphoryl group transfer is not the rate-limiting step of the creatine kinase reaction.

Thus, phosphoryl group transfer is the rate-controlling step of the creatine kinase reaction under physiological conditions, i.e. at 30–37°C, pH 7.4 and [PCr]/[Cr] $\simeq$ 1.0–2.0. However, at lower temperatures or high [PCr]/[Cr] ratios (>5, low ADP), some tendency to shifting of the position of the rate-controlling step was revealed. As a result magnetization transfer allows one to determine true fluxes between phosphocreatine and ATP under conditions close to physiological.

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