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Combination of ^{31}P-NMR magnetization transfer and radioisotope exchange methods for assessment of an enzyme reaction mechanism: rate-determining steps of the creatine kinase reaction

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The theoretical analysis of a reversible enzyme reaction performed in this work shows that the ^{31}P-NMR magnetization (saturation) transfer technique combined with a radioisotope exchange method may potentially provide information on the position of rate-determining step(s). It depends on chemical shifts of NMR signals of nuclei of interest in free and enzyme-bound forms of substrate(s) and product(s) of the reaction. The creatine kinase reaction (MgATP + creatine $\rightleftarrows$ MgADP + P-creatine) has been used as a model. Chemical shifts of ^{31}P in binary, ternary and transitional state substrate-enzyme complexes have been estimated by the variable frequency saturation transfer (VFST) method. This method is based on selective irradiation of numerous points in the spectrum and observation of changes in the intensity of visible line(s) which occur due to chemical exchange between it and lines which are not visible in the routine spectrum. Also, dissociation rate constants of MgADP-containing complexes were determined. Magnetization exchange rates, P-creatine $\rightleftarrows$ [γ-P]MgATP and [β-P]MgADP $\rightleftarrows$ [β-P]MgATP, were compared with radioisotope exchange rates, [γ-^{32}P-MgATP $\rightleftarrows$ P-creatine and [^{3}H]MgADP $\rightleftarrows$ MgATP at different [P-creatine]/[creatine] ratios and at different temperatures. All these exchange rates were close to each other at 30-37°C and [PCr]/[Cr] ratios lower than 2. It is concluded that phosphoryl group transfer is the rate-determining step of the overall creatine kinase reaction under these conditions. However, at lower temperatures (below 25°C) or at high [PCr]/[Cr] ratios ([ADP] < 20 μM) the rate-determining step seems to be shifted toward dissociation of nucleotide substrates from enzyme-substrate complexes, since exchange rates became significantly different. This approach is useful for analysis of mechanism of enzymatic reactions and also can be applied to non-enzymatic reactions and evaluation of small rapidly exchangeable metabolite pools.

Introduction

Creatine kinase (CK) catalyses the net reaction of reversible phosphoryl group transfer from MgATP to creatine (Cr), resulting in formation of MgADP and phosphocreatine (PCr). The following partial exchanges are related to this reaction: ATP–ADP, PCr–Cr, PCR–[γ-P]ATP, and have until recently been measured by a radioisotope tracer method. The classical approach for analyzing the reaction mechanism and the position of the rate-determining steps was a comparison of the rates of different exchanges (e.g., ATP–ADP and PCr–Cr) with labeled substrates [1,2]. During last decade the ^{31}P-NMR magnetization transfer technique has been introduced for investigations of PCr-[γ-P]ATP exchange catalyzed by creatine kinase in vitro [3–8] as well as in living tissues [5,7,10–15]. In general, the rates obtained by this method may be dependent on chemical shifts of nuclei of interest in substrate-enzyme complexes and on the position of the rate-determining step(s) and therefore may be different from radioisotope ex-

Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether) tetraacetate; PCr, phosphocreatine; Cr, creatine; cCr; cyclocreatine; PcCr, phosphocyclocreatine; CK, creatine kinase; Hepes, hydroxyethylpiperazineethane sulfonate.

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change rates, as briefly discussed in our previous papers [5,8,9]. Therefore, a combination of radioisotope tracer and ^{31}P-NMR magnetization transfer methods may provide additional information on the mechanism of enzymatic reactions. Thus, comparison of the exchange rates of PCr-[γ-P]ATP detected with [γ-^{32}P]ATP and ^{31}P-NMR carried out in our previous work [8] showed that phosphoryl group transfer is the rate-determining step of the creatine kinase reaction under physiological conditions and that magnetization transfer is a reliable method for the evaluation of correct values of unidirectional fluxes [8]. The latter was emphasized by Brindle et al. [3,6], who compared the rate of [γ-P]ATP⟷PCr exchange assessed by saturation transfer with those of PCr⟷Cr exchange detected with ^{15}N-labeled creatine [3]. Also, they found that [β-P]MgADP–[β-P]MgATP exchange rates measured by saturation transfer can be close to or different from those detected by radioisotope exchange with [^{14}C]ADP [6], depending on experimental conditions. The effect of saturation loss in reaction intermediates on measured magnetization flux has been discussed [6,9]. nabb,cad

To develop this method further we have studied chemical shifts of creatine kinase-substrate complexes and their dissociation rate constants using the variable frequency saturation transfer (VFST) method which was developed for investigations of papain [15] and dihydrofolate reductase substrate-enzyme complexes [17,18]. In addition, we have compared the rates of the exchanges PCr–[γ-P]ATP and ATP–ADP assessed both by ^{31}P-NMR saturation transfer and radioisotope tracer methods. In general, good agreement between all measured rates was observed at physiological temperatures (30–37°C); lowering the temperature resulted in discrepancies between them, thereby suggesting a shift of the rate-determining step position from phosphoryl group transfer to dissociation of some enzyme-nucleotide complexes.

Part of this work has been published in Ref. 9 as a Lecture delivered at the Soviet-American Symposium on Myocardial Metabolism, held in Baku, U.S.S.R., in September, 1984.

Materials and Methods

^{31}P-NMR experiments

All measurements were done in 10 mm sample tubes of a WM-500 NMR spectrometer (Bruker, F.R.G.) detecting ^{31}P at 202.45 MHz. Typical parameters for accumulation and transformation of routine spectra were as follows: 90° pulse width ranging from 40 to 86 μs depending on sample conductivity (ionic strength) and transmitter attenuator position; repetition time, 5–20 s; acquisition time, 0.2–1.0 s; spectral width 5–10 kHz; memory size, 4–16 K; number of scans, 16–32. The prior Fourier transformation original signal was multiplied exponentially with a broadening factor ranging from 10 to 20 Hz.

Selective saturation [19] of individual phosphorus signals was carried out by the 'Dante' method [20]. In this experiment saturation was attained using short (i.e., 1 to 4 μs) pulses arranged in pulse trains of 1000 to 2000 pulses with 100 to 250 μs intervals between each pulse. Longer periods of saturation were attained by applying several of these pulse trains in sequence with 300 μs delay between each pulse train and an acquisition period. The central band of the radiofrequency power spectrum generated by the 'Dante' sequence was used for the saturation of the signals chosen; the repetition time for the application of short pulses (100–250 μs) was selected to avoid interference by the nearest sidebands of the saturating power spectrum (4–10 kHz off the central band, respectively) with other lines in the spectrum. The power of the saturating pulse was varied by changing the duty time of the saturating sequence (from 0.5% to 2.5%) and the power of the transmitter output (from 0 to 9 dB). Saturation transfer was performed in two modes:

(a) Selective saturation at fixed frequency while varying the time of saturation [19]. Treatment of data obtained in these experiments and calculation of pseudo-first-order rate constants and T_1 values were as described in our previous work [5,8].

(b) The frequency of the selective saturation was varied while the duration of the saturation remained fixed, long enough to reach steady-state distribution of magnetization [3–5 s). This procedure was described earlier [16–18] and will be referred to as variable frequency saturation transfer (VFST).

T_1 was determined by inversion or saturation recovery. Saturation recovery experiments were done by using an aperiodic sequence:

$$90\text{-}t\text{-}90\text{-}t/2\text{-}90\text{-}t/4\text{-}\ldots\text{-}90\text{-}t/2^n\text{-}90$$

In the typical sequence, $t = 1$ s and $n = 10$. This nonselective saturating sequence was followed by a variable delay and a 90° sampling pulse (40 μs).

T_2 was estimated by the Carr-Purcell-Meiboom-Gill method [21,22]. The basic incubation medium contained 20–50 mM Hepes-Na (pH 7.4), 110 mM potassium acetate, 30 mM KCl, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 0.5 mM EGTA-Na, 20% D_2O for field-frequency lock, variable concentrations of ATP and ADP ([ATP] + [ADP] = 8 mM), PCr, Cr ([PCr] + [Cr] = 30 mM) and creatine kinase indicated in the legends to the figures.

Measurements of [γ-^{32}P]ATP–phosphocreatine exchange

This exchange was assayed in the same basic medium as the saturation transfer, using 0.4 mg/ml (approx. 80 IU/ml) of creatine kinase, 8 mM ATP and [PCr] + [Cr]

= 30 mM essentially as outlined in Ref. 8. 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 a position close to equilibrium. The exchange reaction was then initiated by the addition 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 [23]. Such treatment at 0 °C removed about 97% of the ATP and decreased the phosphocreatine content by nor more than 10%. Radioactivity of these samples after separation of charcoal was counted in water in a 'Rack Beta 1215' scintillation counter (LKB, Sweden).

Measurements of [^{3}H]ADP–ATP exchange

This reaction was assayed in essentially the same way as the [γ-^{32}P]ATP–PCr exchange, except for the creatine kinase concentration, which was 0.02 to 0.04 mg/ml (4–8 IU/ml, 0.5–1.0 μM of the active sites). The reaction was initiated by addition of approx. 5 μM of [^{3}H]ADP. Samples (50 μl) were withdrawn at certain time intervals (5 to 600 s at 30–37 °C) and the reaction was terminated by mixing with 50 μl of ice-cold solution (0 °C) containing 0.15 M potassium phosphate (pH 8.0), 20 mM EDTA and 5 mM 2,4-dinitro-1-fluorobenzene. This mixture (2 μl, 10 nmol of total nucleotides) was applied on PEI-cellulose plates ('Merck', F.R.G.) and separated by ascending chromatography using 0.15 M potassium phosphate (pH 8.0). Spots corresponding to ADP and ATP were cut off and their radioactivity was determined in toluene scintillator in a 'Rack Beta 1215' scintillation counter.

This amount of labeled ADP could not markedly disturb equilibrium at [PCr]/[Cr] $\approx$ 1.0, as the equilibrium ADP concentration was about 80 μM and the increase in [ADP] was only by 6%. However, at higher [PCr]/[Cr] ratios, between 2 and 5, when equilibrium [ADP] was from 40 to 16 μM, respectively, the shift in ADP concentration from the equilibrium position is more profound (by 12.5 to 30%). This should result in the appearance of the net flux from ADP to ATP and an increase in the initial isotopic flux by no more than 30% at minimal equilibrium [ADP], assuming linearity between flux and [ADP] ([ADP]/K_m(ADP) = 0.1). Obviously, equilibrium [ATP], [PCr] and [Cr] could not be affected because all of them are hundreds of times higher than [ADP]. Note that chemical equilibrium is established much faster than isotopic equilibrium, since the life-time of ADP is about 0.2 s ($\approx$ [ADP]/flux). Hence, only the initial incorporation of the radioactivity into ATP is overestimated (5 s point), whereas all other time points are unaffected by equilibrium re-establishment. Consequently, analysis of these data in the semilogarithmic plots taking into account several time points allows us to reduce the systematic error, which does not exceed 30% in the extreme case ([PCr]/[Cr] = 5).

Calculation of the rate constants of isotopic exchange

Rate constants were calculated either from initial slopes of the curves of radioactivity incorporation into phosphocreatine (or ATP) or by plotting ln(CPM_∞-CPM_t) vs. time, where CPM_t and CPM_∞ represent the current radioactivity of PCr or ATP at time t and the equilibrium radioactivity at infinity, respectively. From the slopes of the straight lines in semilogarithmic coordinates the sum of the rate constants, $(k_1 + k_2)$ or $(k_1 + k_2')$, can be determined, which are defined at equilibrium as

$$k_1[\text{ATP}]_{eq} = k_2[\text{PCr}]_{eq} = \text{flux}$$

for [γ-^{32}P]ATP–pCr exchange, and

$$k_1[\text{ATP}]_{eq} = k_2'[\text{ADP}]_{eq}$$

for [^{3}H]ADP–ATP exchange, and therefore,

$$k_2 = \text{slope}/(1 + [\text{PCr}]_{eq}/[\text{ATP}]_{eq})$$

and

$$k_1 = \text{slope}/(1 + [\text{ATP}]_{eq}/[\text{ADP}]_{eq}, \text{ respectively.}$$

Enzymes

The MM isoenzyme of creatine kinase was isolated from rat skeletal muscle according to Keutel et al. [24]. The enzyme had high specific activity, ranging from 200 to 300 IU per mg of biuret protein as determined in reverse reaction at 30 °C and pH 7.4 by a coupled enzyme spectrophotometric assay as described earlier [5,8]. Hexokinase and glucose-6-phosphate dehydrogenase were purchased from Sigma.

Other reagents

Nucleotides, creatine and Hepes buffer were obtained from Sigma or Calbiochem (U.S.A.). Phosphocreatine, disodium salt was supplied by Schiapparelli Farmaceutici (Italy) and cyclocreatine was synthesized as outlined earlier [25,26]. [γ-^{32}P]ATP and [^{3}H]ATP were obtained from Amersham (U.K.). The salts were of purest and analytical grades.

Results

Visualization of ADP-containing complexes of creatine kinase by VFST

In previous studies the substrate-enzyme complexes of CK were investigated by using high enzyme and substrate concentrations (both millimolar) [27] and they

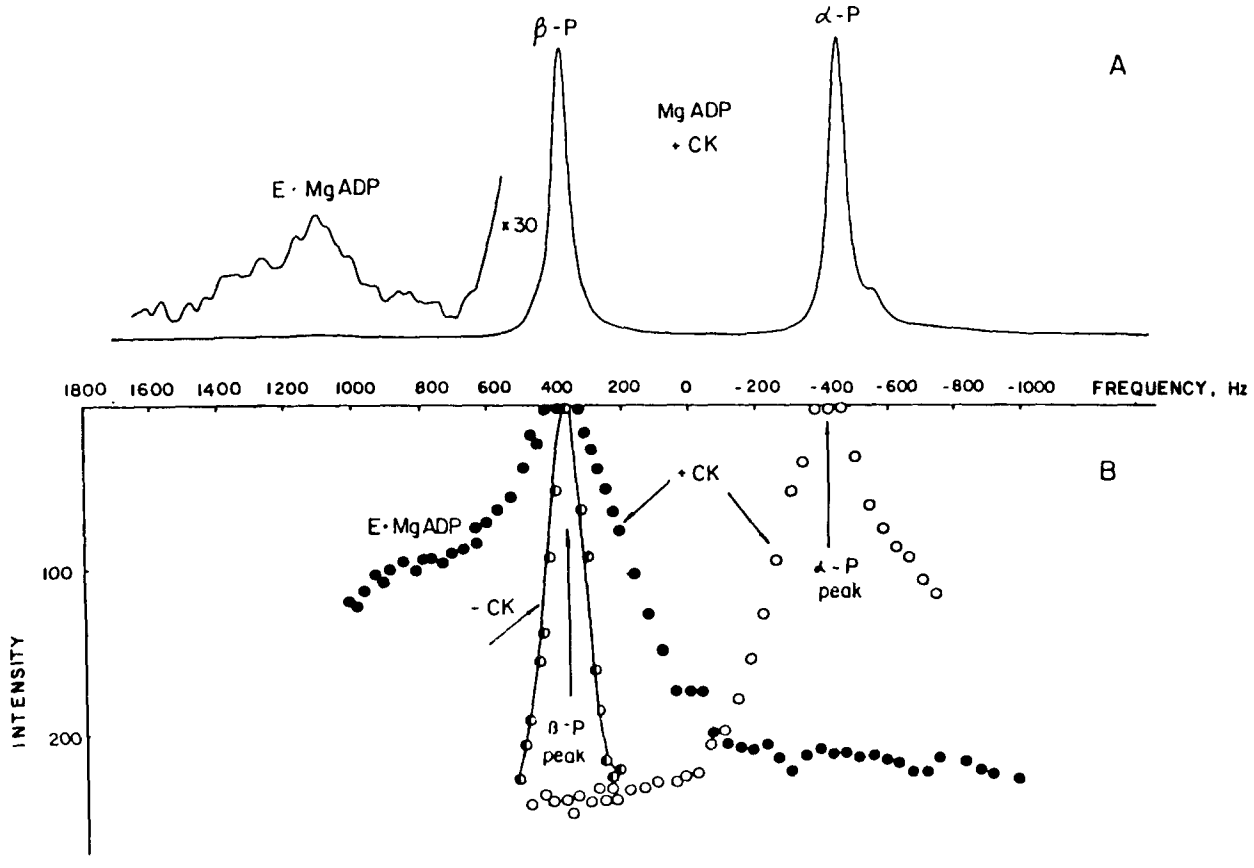


Fig. 1. (A) ^{31}P spectrum of MgADP solution in the presence of 40 mg/ml of creatine kinase. Other components of the incubation mixture are given below. Number of scans, approx. 53000. (B) Intensity of the β-P (MgADP) signal as a function of frequency of the selective saturating pulse (central band of the 'Dante' sequence, see Methods). The frequency of the central band of saturating sequence is not expressed in absolute units but is given as an offset parameter with respect to synthesizer frequency. The total time of saturation was 3 s. The saturating comb consisted of short 1 μs pulses separated by 99 μs intervals so that average power of this pulse train was 50 cps and nearest side-bands were 10 kHz off the central one. The saturating sequence was immediately (300 μs) followed by a 90° (100 μs) sampling pulse. The sample contained 8 mM ADP, 20 mM magnesium acetate, 20 mg/ml creatine kinase (where shown), 1.2 mM dithiothreitol, 0.5 mM EGTA, 70 mM Hepes-Na (pH 7.4) and 20% (v/v) D_2O. Temperature, 30°C.

concern mostly binary and dead-end ternary and quaternary complexes at low temperatures (0–10°C). However, the chemical shifts of β-P of MgADP in ternary productive complexes as well as their kinetic properties at physiological temperatures (30–37°C) remain unknown.

To solve these problems we have used a method for visualization of reaction intermediates which cannot be easily observed in routine NMR spectra because of their small concentrations and broad NMR lines (see Refs. 16–18). For this purpose the selective saturating pulse generated by the 'Dante' sequence was moved stepwise through the spectrum (i.e., the frequency of the irradiating pulse was varied) over a preselected frequency range which was followed by a nonselective sampling pulse. Fig. 1 shows results of such experiment with the system containing MgADP alone. A decrease in the intensity of the β-P and α-P signals was observed when the frequency of the saturating pulse was close to the β-P or α-P peak, respectively, due to the direct partial or complete saturation of these signals. The dependence of the observed signal intensity on the frequency of the saturating pulse appears to be bell-shaped, with the same maximum as in the normal spectrum.

However, with creatine kinase a decrease in the signal from β-phosphate of the free MgADP was observed when the saturating pulse frequency was far downfield of β-P (centered approximately 600 Hz downfield) which was not observed in the absence of the enzyme. This effect can be explained by saturation transfer from binary complex(es) of E · MgADP invisible in the spectrum. Indeed, long-term data accumulation of the spectrum of the MgADP sample containing a high creatine kinase concentration (40 mg/ml) reveals a broad line located approx. 700 Hz downfield of the β-P signal of free MgADP, in agreement with the earlier studies of Rao and Cohn [27].

The VFST method was also applied to a system containing the quaternary transitional state analog complex, Cr-NO_3^--E-MgADP. In this case saturation trans-

TABLE I

Chemical shifts of [β-P]MgADP in complexes with creatine kinase and their dissociation rate constants

Temperature, 30°; pH 7.4. Values are approximate.

Complex	Chemical shift of β-P [a] (ppm)	Dissociation rate constant (s^{-1})
E·MgADP	+3	150
Cr·E·MgADP	+3	60
Cr·NO_3·E·MgADP	+1	60
PCr·E·MgADP and/or Cr...PO_3^-...MgADP·E	−6	n.d. [b]

[a] Chemical shift is given with reference to the β-P of free MgADP.
[b] n.d., not determined.

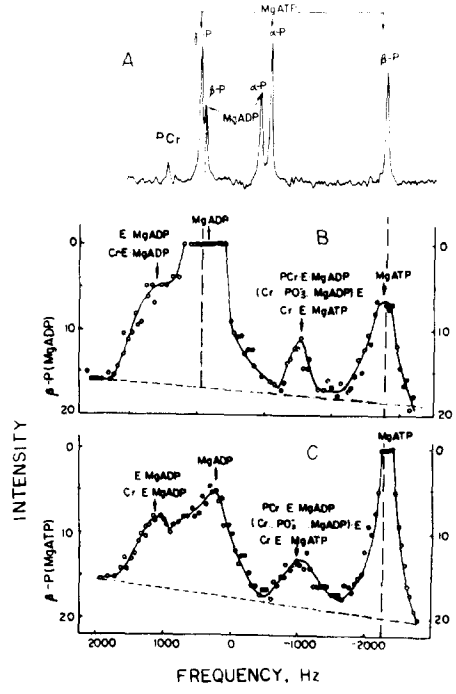
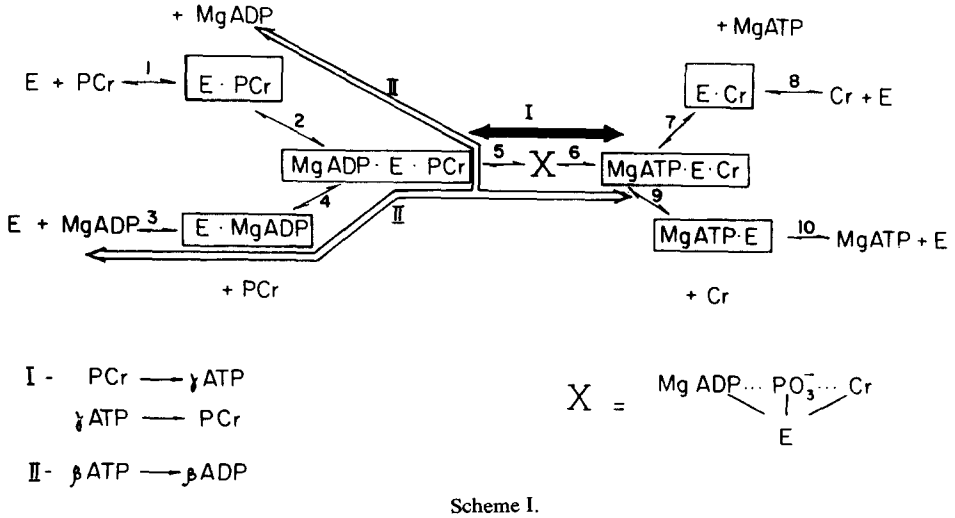


Fig. 2. Analysis of the complete creatine kinase system by variable frequency saturation transfer. (A) ^{31}P-NMR spectrum of mixture of creatine kinase substrates in the presence of catalytic amounts of creatine kinase. Composition of incubation mixture is as below. (B, C) Intensities of β-P signals of MgATP (B) and MgADP (C) are given as a function of the frequency of a selective saturating pulse. All NMR parameters were the same as described in Fig. 1 except for the power of the saturating pulse. The sample consisted of approx. 4.0 mM MgATP, 3.5 mM MgADP, less than 1 mM phosphocreatine, 96 mM creatine and 20 mg/ml creatine kinase in 75 mM Hepes-Na (pH 7.4). All other components were identical to those reported for Fig. 1. Temperature, 30°C.

fer also occurred from the region located approx. 1 ppm upfield of β-P signal of MgADP (not shown). Most probably this phenomenon can be attributed to the exchange between the quaternary complex in question and free MgADP. All these data are in good accordance with those obtained by Rao and Cohn [27] by direct accumulation of ^{31}P-NMR spectra. Using this approach (VFST) we have also found that in binary E-ADP and dead-end ternary Cr-E-MgADP complexes, β-phosphoryl groups have the same chemical shifts as E-MgADP (see Table I).

An equilibrium mixture of all substrates involved in the creatine kinase reaction in the presence and in the absence of the enzyme was investigated by the VFST method. As seen in Fig. 2B the β-P signal intensity of MgADP decreases due to direct saturation of β-P of MgADP and via saturation transfer from the β-P of MgATP due to chemical exchange. In addition, transfer was observed from a region centered between [β-P]MgATP and [β-P]MgADP (approx. 6 ppm upfield of [β-P]MgADP). Simultaneously, some decrease in β-P signal intensity of MgATP was also observed when the saturating pulse was placed at the same frequency range (Fig. 2C). These exchanges were not observed in the absence of the enzyme. These data imply that signals of



Scheme I.

the β-P of MgADP and MgATp in the ternary productive complexes, Pcr-E-MgADP and/or creatine-E-MgATP, as well as in some intermediates, e.g., the hypothetical transitional state complex (creatine... PO_3^- ... MgADP-E [28–30], can be in this spectral range (see Scheme I). Involvement of the binary E-MgATP complex is hardly possible, since we did not find this effect in a binary system consisting of MgATP and CK (not shown). Moreover, according to Rao and Cohn [27], the chemical shift of the β-P of MgATP was not altered substantially (less than 0.5 ppm) in this binary complex. 2;Sch. 1

In order to discriminate between ternary and transitional state complexes, a creatine analog, cyclocreatine (cCr), was used instead of creatine as a component of the complete creatine kinase system. Phosphocyclocreatine (PcCr) is poor substrate of creatine kinase, with V_{max} equal to approx. 1/100 of the V_{max} with PCr [25,26] and, therefore, to 1/20 of the V_{max} with creatine (in the opposite direction). Hence, the overall equilibrium flux through creatine kinase with the PcCr/cCr pair should be at least 20-times slower than with natural substrates, as the upper limit of the flux is determined by the smallest V_m. Assuming that quasi-equilibrium mechanism of the reaction is maintained, since the K_m values for the nucleotides' substrates are not affected when PcCr and cCr are used instead of PCr and Cr [26], we may suppose that expected decrease in the overall flux is due to the reduction of the rate of phosphoryl group transfer. Consequently, at the same enzyme concentration, conversion of ternary complexes (PcCr-E-MgADP via cCr... PO_3 ... MgADP · E to cCr-E-MgATP) may be much slower. Therefore, it should be easier to detect by VFST separately the exchange of MgADP-containing complexes with free MgADP (MgADP ⟷ MgADP · E · PcCr) and MgATP-containing ones with MgATP (MgATP ⟷ MgATP · E · cCr) without interference between them. As seen in Fig. 3, magnetization transfer was observed from β-P of E-MgADP (approx. 3.5 ppm downfield of β-P (MgADP)) and the region located approx. 5 ppm upfield of β-P of free MgADP signal to the β-P of free MgADP. Meanwhile, no transfer was observed from this region to the β-P of MgATP as well as between the β-P's of MgADP and MgATP, and between the γ-P of MgATP and PcCr. 3

Indeed, in accordance with the assumption presented above, in this system overall fluxes are not detectable under these conditions. However, the partial flux to [β-P]MgADP from intermediate(s), signals of which are in the same spectral region as those observed with natural guanidine substrates (see Fig. 2), was measurable (see Fig. 3). Since transfer from region corresponding ternary complexes occurred only to the β-P of MgADP and not to the β-P of MgATP, this suggests that the ternary MgATP-E-cCr (or Cr) complex is not responsible for transfers taking place from this part of the spectrum. Otherwise, the 'saturated' β-P of MgATP

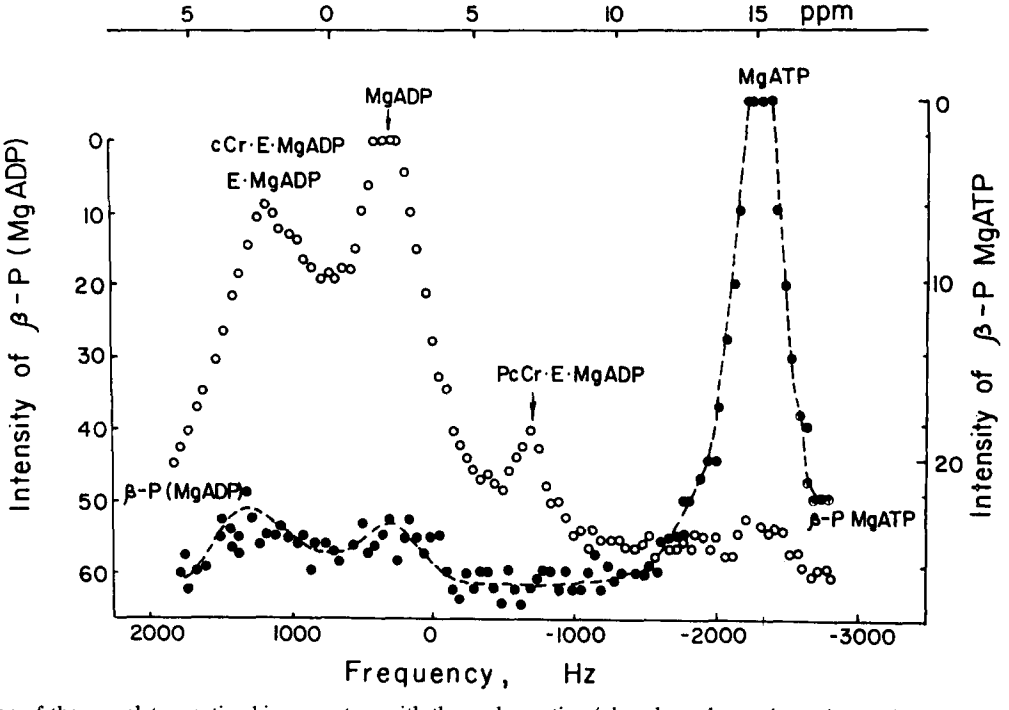


Fig. 3. Investigation of the complete creatine kinase system with the cyclocreatine/phosphocyclocreatine pair as substrates. NMR parameters were as those given in Fig. 1. The sample contained approx. 5 mM MgATP, 10 MgADP, 10 mM phosphocyclocreatine, 90 mM cyclocreatine and 12 mg/ml of creatine kinase in 72 mM Hepes-Na (pH 7.4). All other components were as listed in the legend to Fig. 1.

in a ternary complex should be easily transferrable to the β-P of free MgATP due to its relatively fast dissociation.

Another interesting question concerning NMR properties of creatine kinase reaction intermediates refers to chemical shifts of the transferrable phosphoryl group of ATP (γ-P) and PCr. Fig. 4 shows the dependence of the intensities of signals from these groups in the presence of CK on the frequency of the saturating pulse. Lines arising in such a 'spectrum' overlap substantially in both cases when intensities of PCr (Fig. 4A) or [γ-P]ATP (Fig. 4B) signals are monitored. Therefore, it is unlikely that signals of some intermediates will be observed if they are located between [γ-P]MgATP and phosphocreatine. Also, the exchange rate of PCr⟷[γ-P]ATP did not depend on the power of saturating pulse applied (within the limits of selectivity). This suggests that, if the transferrable group of a complex with the enzyme has a line separated from those of the γ-P of MgATP and PCr, this hypothetical line is not in close vicinity to them. Otherwise, we could observe a progressive increase in observed rate of exchange, PCr → [γ-P]MgATP, as a power of the saturating pulse raised.

Estimation of dissociation rate constants of creatine kinase complexes

We attempted to estimate the kinetic properties of the aforementioned complexes. With this aim, the invisible line belonging to a complex was irradiated by a selective saturating pulse of variable duration at the resonance frequency found for this line. However, since we could not directly observe the degree of saturation of

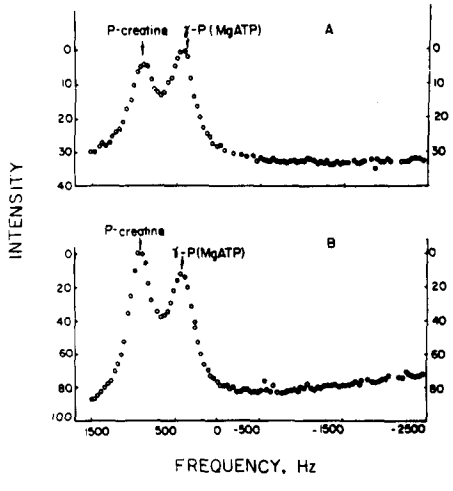


Fig. 4. Dependence of signal intensities of γ-P of MgATP (A) and P-creatine (B) on saturating pulse frequency in the complete creatine kinase system. Parameters of the saturating train were similar to those given in legend to Fig. 1, except for saturating power, which was 2.3-times higher. The sample comprised 10 mM phosphocreatine, 10 mM creatine, 5 mM MgATP and 20 mg/ml of creatine kinase in 70 mM Hepes-Na (pH 7.4). Temperature, 30 °C.

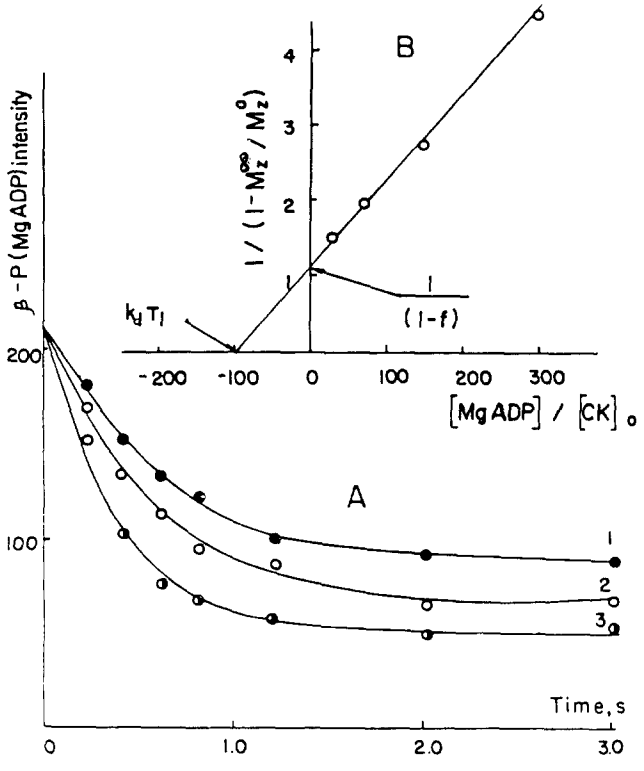


Fig. 5. Estimation of the dissociation rate constants of creatine kinase-MgADP complex(es). (A) Dependence of β-P of MgADP signal intensity on time of saturation of the β-P line of the E-MgADP complex at increasing powers of saturating pulse. The central band arising as a result of 'Dante' sequence application was placed 600 Hz downfield of the β-P line of free MgADP. Powers expressed in cycles/s were 1, 50; 2, 62; 3, 75. Definition of power unit is given in the text to Table II. The experiment was carried out in a medium containing 8 mM MgADP and 5 mg/ml of creatine kinase in 70 mM Hepes-Na (pH 7.4) at 30 °C. (B) Steady-state saturation transfer. Dependence of the $(1/(1 - M_{z\infty}/M_{z0})$ vs. $[MgADP]/[E]_0$ ratio. Total time of saturation was 5 s. The sample contained 7.5 mM MgADP and variable concentrations of creatine kinase (1–20 mg/ml) in 70 mM Hepes-Na (pH 7.4). Temperature, 23 °C.

the invisible signal, we used two indirect approaches. First, the power of saturating pulse was progressively increased up to the limit of selectivity required. The average power of the saturating pulse generated by the 'Dante' sequence was varied as described in Materials and Methods. Fig. 5 demonstrates results of such an experiment with the system containing MgADP and CK ($[MgADP] \gg [E]_0$). Fig. 5A shows that the curve of time-dependence of intensity of the β-P line of free MgADP shifted down as the saturating pulse power rose. As seen in Table II the calculated (observed) rate constant increases as the saturating pulse power increases and there is no tendency to level-off this dependence within the limits of selectivity. This latter result implies that complete saturation of the enzyme-substrate complex was not attained with the power levels applied. The lack of complete saturation is probably due to the broad line of th E-MgADP complex. There-

TABLE II

Observed rate constant of dissociation of MgADP from binary complex(es) with creatine kinase and steady-state magnetization of [β-P]MgADP as a function of irradiation power of selective pulse

Data are taken from experiments similar to those shown in Fig. 5A. The power of irradiating pulse was varied by changing the duty time of the Dante sequence and/or by changing transmitter output, and is expressed in cycles per second (c/s). Power was calculated by the formula: (duty time, %)×1 s/100×(duration of 360° pulse), and means number of 360° pulses per second. Other conditions are given in legend to Fig. 5A.

Power (c/s)	50	62	75	100
k^1_{obs}/E_0 ($s^{-1}\cdot mmol^{-1}$)	9.5	11.2	16.9	21.5
$M_{z\infty}/M_{z0}$ +CK	0.53	0.65	0.73	0.83
−CK	0	0	0.1	0.18

fore, this approach provided only a lower limit of the values of the rate constants involved in this exchange.

To avoid problems connected with uncertainty in the degree of saturation of the invisible signal, we have used another approach. For the case of incomplete saturation of a selected line, a relationship can be derived from equation obtained by Campbell et al. [31], which for steady-state magnetization ($M_{z\infty}$) in double-reciprocal form is (see Appendix 2):

$$1/(1-M_z^\infty/M_z^0) = 1/(1-f) + \frac{[\text{MgADP}]/E_0}{k_d T_1(1-f)} \quad (1)$$

where f is degree of incompleteness of saturation and $(1-f)$ is degree of saturation, respectively; k_d, dissociation rate constant and T_1, spin-lattice relaxation time of [β-P]MgADP.

If T_1 for MgADP is known, dissociation rate constant of E-MgADP can be calculated by plotting $1/(1-M_z^\infty/M_z^0)$ as a function of the [MgADP]/[CK] ratio at saturation of the β-P of E-MgADP. Results from this experiment are presented in Fig. 5B. In agreement with theory (Eqn. 1), experimental points obey a linear relationship. Taking T_1 for the β-P of MgADP equal to 1.8 s, k_d values were calculated.

In general, there is good correlation between the data obtained by the two methods described above. Table I summarizes properties of substrate- enzyme complexes of CK, viz., chemical shifts of their phosphorus signals and their dissociation rate constants. The chemical shift of the β-P of MgADP is the most sensitive to the changes in chemical environment induced by binding with the enzyme. Actually, the β-P signal of MgADP shifts by 3–3.5 ppm downfield in binary and abortive ternary complexes and by 5–6 ppm upfield in ternary productive and/or transitional state complexes.

The rate constant of MgADP release from complexes listed in the table is high for binary complex, E-MgADP ($k_d \approx 150\ s^{-1}$), and 2–3-times smaller for dead-end ternary and tertiary complexes. It is interesting that dissociation rate constant of E-MgADP is comparable with enzyme turnover in the reverse direction (PCr → ATP), which is about 160 s^{-1} and 4–5-times faster than the turnover in the forward direction, since V_m(reverse)/V_m(forward) ≈ 4.4–5.8 [5,8]. Consequently, it is faster than the overall turnover rate under equilibrium which is limited by the lowest V_m and about 40 s^{-1} at 30°C and pH 7.4 [5,8]. Thus, dissociation of MgADP seems unlikely to be the rate-limiting step of the creatine kinase reaction at pH 7.4 and 30°C.

Influence of binding of PCr, MgADP and MgATP to creatine kinase on the relaxation parameters of ^{31}P

According to the theoretical consideration given in Appendix 1, under conditions of fast exchange when

TABLE III

The effect of creatine kinase on relaxation parameters of ^{31}P in MgADP, MgATP and PCr

Parameter	Conditions ($[E]_0/[S]_0$)	Line in the spectrum		
		β-P of MgADP	γ-P of MgATP	PCr
T_1 (s)	0 (no CK)	1.35	1.83	3.52±0.005
from inversion recovery [a]	0.1	1.32	1.72	2.69±0.02
T_1 (s) from saturation transfer [b]	0.02–0.05	n.d.	1.55± 0.05	2.83±0.29
T_2 [a] (ms)	0	98	96	220
	0.05	28	41	n.d.
	0.125	20	18.5	75

[a] T_2 times were measured by CPMG method [21,22] and T_1 by inversion recovery. T_1 and T_2 for PCr were determined in 98% D_2O (except for saturation transfer experiments, where $[D_2O] = 20\%$) to avoid the isotope splitting of PCr signal which occurs in mixtures of H_2O and D_2O [33]. Concentrations of MgADP and MgATP were 4 mM and that of PCr was 5 mM. Temperature, 30°C; pH 7.4.

[b] To obtain these values experiments were performed in which saturation transfer from [γ-P]MgATP to PCr and from [β-P]MgATP to [β-P]MgADP was monitored in the presence of an equilibrium mixture of all substrates.

lines of free and bound forms collapse due to the small chemical shift difference between them, the observed T_1 can become shorter in the presence of the enzyme. To test this assumption, T_1 and also T_2 values of ^{31}P in PCr, MgATP and MgADP were measured in the presence and absence of the enzyme (Table III) and the former were compared with those obtained by saturation transfer. It is seen that the observed T_1 was shortened after CK addition only for PCr at an $[E]_0/[S]_0$ ratio of 0.1, whereas T_1 values for the γ-P of ATP and the β-P of MgADP were not affected by binding to the enzyme. Also, T_1 values obtained from saturation transfer experiments at $[E]_0/[S]_0 = 0.02–0.05$ were close to those determined above in binary systems (see Table III).

Thus, the shortening of the observed T_1 value for PCr induced by binding to CK points to a smaller T_1 for it in the binary E · PCr complex. Furthermore, the absence of any detectable effect of CK on T_1 values for the γ-P of MgATP and the β-P of MgADP points to less significant changes in T_1 in binary nucleotide-enzyme complexes.

Observable T_2 values were more sensitive to binding to the enzyme and were reduced 5-times for the terminal phosphates of nucleotides and 3-times for PCr at $[E]_0/[S]_0 = 0.125$. Since conditions of slow exchange are fulfilled only for the β-P of MgADP (due to the large chemical shift difference), the rate constant of dissociation of binary complex can be estimated only for MgADP using the formula:

$$k_\alpha = \frac{1/T_2(+CK) - 1/T_2(-CK)}{[E]_0/[MgADP]_0} = 280–300\ s^{-1}$$

This k_d value is approximately twice that found in saturation transfer experiments. However, since the β-P signal of MgADP is a doublet due to spin–spin interaction with α-P, T_2 values can be underestimated [29] (although $2\tau = 1$ ms was taken as minimal to reduce effect of coupling) and, therefore, k_d can be somewhat overestimated. Hence, the estimation of k_d in the range 160–300 s^{-1} seems to be reasonable.

Comparison of the rates of exchange of phosphocreatine–[γ-P]ATP and [β-P]ADP–[β-P]ATP measured by saturation transfer

Data reported in previous sections together with those available in the literature [5,6,8] show that PCr–[γ-P]ATP and [β-P]ADP⟷[β-P]ATP exchanges catalyzed by creatine kinase which are detected by ^{31}P magnetization transfer can depend on different steps of the reaction mechanism because of the different NMR properties of the reaction intermediates (see Appendix 1). In fact, the magnetization transfer pathway for ADP–ATP exchange should involve steps of MgADP association with free enzyme (step 3) and with binary

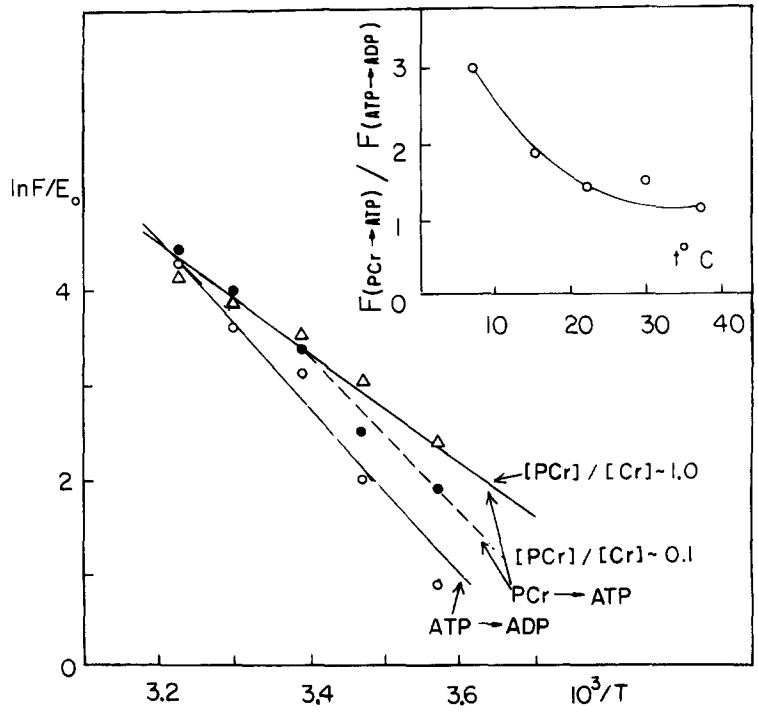


Fig. 6. Temperature dependences of magnetization fluxes, PCr → [γ-P]MgATP and [β-P]MgATP → [β-P]MgADP, measured by the saturation transfer technique. Inset shows ratio of these fluxes as a function of temperature. Conditions are given in Materials and Methods.

complex E · PCr (step 2) as well as the steps of ternary complex transformation (5&6, see Scheme I), since the β-P lines of all these intermediates have markedly different chemical shifts with respect to that of the β-P of free MgADP (Appendix 1, case 1). The opposite situation applies for PCr⟷[γ-P]ATP exchange. In this case, according to our data and those of Rao and Cohn [27], chemical shifts of the phosphorus of PCr and the γ-P of MgATP are only slightly affected by their incorporation into binary and ternary complexes with creatine kinase. Therefore, PCr in free and bound forms should give only a single line as well as [γ-P]MgATP (see Fig. 4). For this reason, saturation (magnetization) transfer between [γ-P]MgATP and PCr is presumably determined by the steps of the chemical transformation of the ternary complexes (Appendix 1, case 2), i.e., by phosphoryl group transfer, whereas the steps of binary and ternary complex formation and breakdown are masked and do not affect this process (see Scheme I).

Thus, the only common step for both exchanges, PCr⟷[γ-P]MgATP and [β-P]MgADP⟷[β-P]MgATP, is phosphoryl group transfer. Therefore, one can predict that the flux [γ-P]MgATP → PCr should be faster than that of [γ-P]MgADP → [γ-P]MgATP; both are measured by magnetization transfer. In fact, measuring these fluxes by saturation transfer in the system with a relatively high [ADP]/[ATP] ratio (about 0.15, [ADP] = 1.2 mM), we found that the ATP → PCr flux is close to the ATP → ADP flux at 30–37 °C (see also ref. 6) and faster at lower temperatures, with the ratio of fluxes reaching 3.0 at +8 °C (Fig. 6). This result is in agreement with the conclusion that MgADP dissocia-

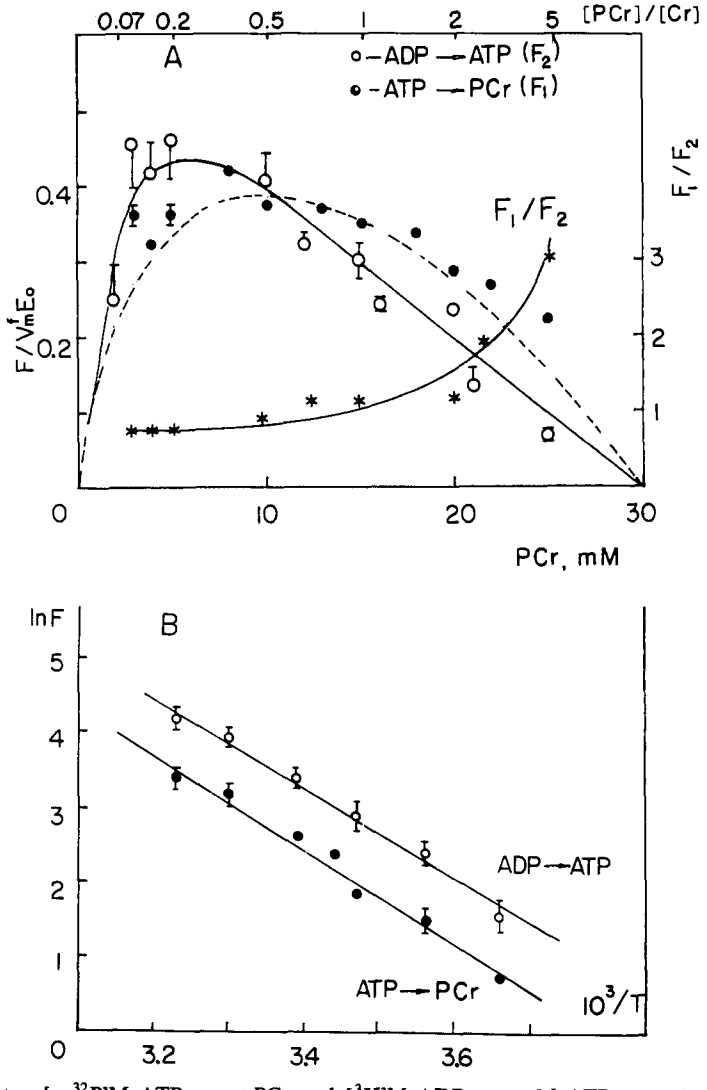


Fig. 7. Radioisotope exchange rates, [γ-^{32}P]MgATP⟷PCr and [^{3}H]MgADP⟷MgATP, as a function of [PCr]/[Cr] ratio (A) and temperature (B). (A) Dotted line represents theoretical curve calculated on the basis of known kinetic constants [5,8]. Flux value is normalized to maximal activity of the enzyme reflecting the amount of active CK. Temperature, 30 °C. (B) Fluxes were measured at fixed [PCr]/[Cr] ratio equal to approx. 1.0. Some vertical shift of Arrhenius plots for different exchanges is due to somewhat different activities of the enzyme used in these experiments. In (A) these fluxes normalized to the maximal activity of the enzyme are very close at [PCr]/[Cr] = 1.0.

tion from enzyme-substrate complexes is not the rate-limiting step of the reaction at 30–37 °C as originally proposed by Morrison and Cleland [1], but can be rate-limiting at lower temperatures. This assumption is in accordance with the data of Rao and Cohn, who found that phosphoryl group transfer is not rate-limiting at +4 °C [25].

Other possible reason for such difference between fluxes (PCr → ATP and ATP → ADP) is recovery of magnetization (loss of saturation) of [β-P]MgADP in substrate-enzyme complexes (e.g., E · MgADP and PCr · E · MgADP), as was pointed out by Brindle and Radda [6]. These two factors, 'kinetic' and 'relaxational', do not exclude each other and may reduce ATP → ADP flux as compared with PCr → ATP flux.

Radioisotope exchange rates as compared with magnetization transfer rates

Fig. 7 demonstrates dependences of isotope exchange rates, [γ-^{32}P]ATP–PCr and [^{3}H]ADP–ATP, on the [PCr]/[Cr] ratio at 37 °C (Fig. 7A) and on temperature at a fixed [PCr]/[Cr] ratio (1 : 1, Fig. 7B). Dependences of both exchange rates were bell-shaped and close in the range of [PCr]/[Cr] ratios from 0.1 to 1.0 (ADP concentrations were from 0.8 to 0.08 mM). However, at higher [PCr]/[Cr] ratios (lower [ADP]), the rate of PCr–ATP exchange exceeded that of ATP–ADP exchange; the ratio of fluxes reached 3 at [PCr]/[Cr] ratio about of 5.0. This phenomenon can be associated with a shift of the rate-determining step from phosphoryl group transfer to ADP association/dissociation step(s) due to decreasing free [MgADP] concentration below 80 μM. In fact, PCr–ATP exchange is less dependent on ADP binding, since the ADP required for this process can be formed from non-labeled MgATP (+Cr in forward reaction). Then ADP either can be rephophorylated in the reverse reaction or may leave its binding site, making it available for the next ATP molecule (labeled or not) binding, and the cycle is repeated. In other words, this stage of ADP association can be replaced by steps of ATP binding and its dephosphorylation. In contrast, for exchange between ADP and ATP, ADP association is obligatory (see Scheme I).

Arrhenius plots of the rates of these exchanges determined at [PCr]/[Cr] ≈ 1.0 have the same slopes, but the straight line for ATP → PCr exchange is shifted downward. This means that both processes have the same temperature coefficient ($\Delta E_a \approx 13$ kcal/mol) and ADP → ATP flux is systematically higher than that for ATP → PCr. However, the latter is a result of different specific activities of the enzyme used for measurements of ADP → ATP and ATP → PCr exchanges and the presentation of flux values referred to mg of enzyme protein. In other words, the rates of these exchanges are identical in all temperature range tested and therefore, both exchanges are controlled by common step(s) of the reaction, most probably by phosphoryl group transfer.

These data and this conclusion are in apparent contradiction with saturation transfer data according to which lowering the temperature leads to an increase in the ratio of fluxes, $F(\text{PCr} \rightarrow \text{ATP})/F(\text{ATP} \rightarrow \text{ADP})$, from 1 to 3 (see Fig. 6). In order to clarify this question, temperature dependences of ADP → ATP exchange rates determined by saturation transfer (β-P of MgADP saturation) and the radioisotope tracer method were compared (see Fig. 8). As can be seen, magnetization and isotope fluxes are identical at 25–37 °C. However, at lower temperatures magnetization flux became 3-times slower than the isotope flux. This difference cannot be explained by kinetic reasons, since the true magnetization flux cannot be lower than isotopic (see Appendix 1). However, it is possible if there is recovery

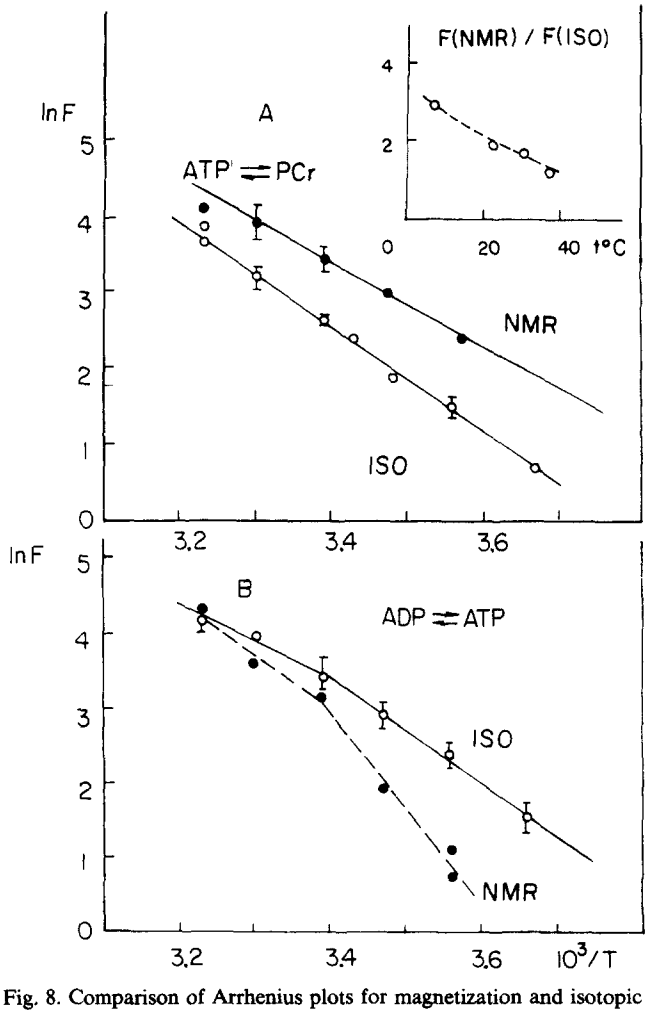


Fig. 8. Comparison of Arrhenius plots for magnetization and isotopic fluxes catalyzed by creatine kinase. (A) Exchange between PCr and MgATP. [PCr]/[Cr] ratio is about 1.0. The inset shows ratio of magnetization and isotopic fluxes as a function of temperature. (B) Exchange between MgADP and MgATP. Isotopic flux was measured at [PCr]/[Cr] = 1.0 but magnetization flux at [PCr]/[Cr] = 0.1, i.e., at a relatively high ADP concentration (about 1.0 mM).

of magnetization (loss of saturation) due to spin-lattice relaxation when a 'magnetic' label passes through reaction intermediates such as E · MgADP, PCr · E · MgADP (see Appendix 1, and Ref. 6). A similar phenomenon has been observed by Brindle and Radda [6], who compared magnetization flux ADP → ATP with isotopic flux [^{14}C]ADP → ATP; the latter was higher at a high [ATP]/[ADP] ratio.

Also, it cannot be ascribed to different [PCr]/[Cr] ratios at which magnetization and isotope tracer fluxes were measured; the former was detected at [PCr]/[Cr] = 0.1, and the latter at 1.0. In fact, they are close at [PCr]/[Cr] = 0/1 (high [ADP]) and 1.0 (low [ADP]) (see Fig. 7). Thus, ATP–ADP exchange rates assessed by saturation transfer can be significantly lower than the real ones (isotopic exchange) under a variety of experimental conditions (low temperature, low [ADP] and pH [6]) and, hence, should be excluded from further consideration.

Discussion

Using the VFST technique we have estimated the chemical shifts of several enzyme-substrate complexes of creatine kinase (see Table I). The chemical shift of the β-phosphoryl group of MgADP was the most sensitive to binding to the enzyme. The signal of this group was shifted downfield by approx. 3 ppm when it was in the E-MgADP or Cr-E-MgADP complexes and by 1 ppm upfield in the quaternary dead-end transitional state analog complex Cr-NO_3-E-MgADP (Table I). In contrast, the chemical shifts of the phosphoryl group of [γ-P]ATP and PCr were not significantly altered by binding to CK. These findings are in good agreement with the direct observations of Rao and Cohn [27] and are complementary, since they functionally confirm the assignments of the resonances observed. In addition, in the present study a resonance was detected by VFST which was consistent with the β-P of MgADP in its active ternary PCr-E-MgADP and/or transitional state (Cr ... PO_3^- ... MgADP-E) complex, the signal of which is located between free [β-P]MgADP and [β-P]MgATP lines approx. by 6 ppm upfield of free [β-P]MgADP resonance.

The dissociation rate constants of some MgADP-containing complexes were estimated in this study using the conventional saturation transfer technique. The dissociation rate constant for the binary E-MgADP complex was about 150 s^{-1} and less than 50 s^{-1} for the ternary dead-end Cr-E-MgADP and transitional state analog, Cr-NO_3-E-MgADP. The dissociation rate constant for E-MgADP determined from the dependence of $1/T_2$ for the [β-P]MgADP line on CK concentration was approx. 300 s^{-1}. The latter value is the upper limit, since the shortening of T_2 was ascribed exclusively to the influence of chemical exchange. The maximal overall turnover of CK under equilibrium conditions at 30 °C is approx. 40 s^{-1}. Therefore, it is reasonable to conclude that the MgADP dissociation step from the enzyme complexes is not rate-limiting in this reaction at this temperature.

Previous studies attempting the determination of the enzyme-substrate complexes relied on the direct observation of the enzyme-substrate complex at high (millimolar) concentrations [27] and on the functional identification of enzyme-substrate complexes by the VFST method. Using VFST we were able to determine the chemical shifts of ADP-containing complexes in relatively dilute (< 0.1 mM) enzyme solutions using their active flux pathways as fingerprints. These relatively low enzyme concentrations prevented interference by other enzyme contamination, which usually resulted in a rapid hydrolysis of ATP, permitting these experiments to be conducted at higher temperatures (up to 37 °C) than was possible with concentrated enzyme solutions [27]. However, the VFST method is limited in its application, since several conditions must be met before it can be used effectively. These conditions include: (a) the chemical shift of line of the substrate-enzyme complex being sufficiently different from that of free substrate to permit selective irradiation; (b) the $1/T_1$ of the free species being of the same order or smaller than the pseudo-first-order rate constant of the exchange; (3) the line of the complex being sufficiently narrow to permit significant saturation with reasonable saturation powers. One of the major advantages of this technique is the functional verification of the peak position by observing where and how fast magnetization was transferred to the free species. Utilizing this type of technique, the chemical shifts and kinetics properties of many enzyme substrate complexes or other small substrate pools may be determined at relatively low concentrations when direct observation of the NMR signals is difficult.

We have shown that the approach based on comparison of magnetization fluxes with respective isotopic ones can provide valuable information on the mechanism of enzymatic (and, possibly, nonenzymatic) reactions. In general, it depends on chemical shifts of nuclei of interest in free forms of substrates and products and in their complexes with the enzyme. If complexes have peaks distinct from those for free forms and from each other (large chemical shift difference), then magnetization transfer gives the same information as isotope tracer under appropriate conditions (see Appendix 1). In contrast, if the chemical shifts of lines corresponding to complexes are close to those of free substrate or product so that these lines are collapsed, then the pathway of magnetization transfer is different from that of isotopic label and, therefore, depends on only certain steps of the reaction mechanism (see Appendix 1).

This is especially evident for [γ-^{31}P]MgATP-PCr magnetization transfer catalyzed by creatine kinase,

since PCr and its complexes have one common line. The same is true for [γ-P]MgATP in free and bound forms. Therefore, in this case magnetization transfer depends only on the stages of chemical conversion (steps 5 and 6 in the Scheme I), unlike isotopic tracer transfer. Consequently, comparison of magnetization and isotopic fluxes may give an idea of how fast or slow steps of association and dissociation of substrate-enzyme complexes are and may allow one to estimate the position of rate-determining step as was done in this work and in Ref. 8. An intermediate situation arose with ATP–ADP exchange: the chemical shift of the β-P peak of MgADP was significantly altered when MgADP was bound with the enzyme in binary and ternary complexes. For this reason magnetization transfer between ATP and ADP was dependent not only on phosphoryl group migration but also on dissociation/association of MgADP from/to the enzyme (steps 2 and 3 in Scheme I) and independent of MgATP binding steps. Moreover, involvement of additional intermediates in the magnetization transfer pathway may facilitate loss of saturation (excitation), resulting in underestimation of the flux value, as demonstrated by Brindle and Radda [6] and confirmed in this work.

Analysis of isotopic and magnetization exchanges catalyzed by creatine kinase (see above) implies that phosphoryl group transfer is the rate-determining step under physiological temperatures (30–37 °C) and [PCr]/[Cr] ratios (from 0.1 to 1.0), in agreement with early finding of Morrison and Cleland [1], confirmed later by presteady-state kinetics data [29,30]. However, at higher [PCr]/[Cr] ratios (above 2), control may be shifted to the steps of substrate-enzyme complex formation and breakdown as the rate of PCr–[γ-P]ATP magnetization exchange exceeded that of isotopic (see Ref. 8) which, in turn, was faster than [^{3}H]ADP–ATP (Fig. 7). A possible candidate for that is MgADP dissociation/association step(s), which can control the overall reaction at the low MgADP concentrations (< 40 μM) existing at high [PCr]/[Cr] ratios. Also, a shift of control seems to take place during lowering of temperature. However, this cannot be ascribed to the step of MgADP binding as was proposed earlier [8], because of the similarity of the temperature dependences of the isotopic exchanges, ATP–PCr and ADP–ATP. Taking into account that the magnetization flux, PCr–ATP, became higher than the analogous isotopic one as the temperature decreased (see Fig. 8 and Ref. 8), it is logical to assume that some other step(s) of the reaction is(are) responsible for this phenomenon. Most probably, it is a process of MgATP association/dissociation, since this step is common for both isotopic exchanges and does not influence magnetization transfer from [γ-P]MgATP to PCr (see Appendix 1 and Ref. 8). This conclusion is compatible with the data of Rao and Cohn [27], who found that phosphoryl group transfer is not rate-limiting at low temperatures (+4 °C) and contradict presteady-state kinetic data [29,30].

The temperature dependence of isotopic and magnetization fluxes deserves special consideration. In general, the apparent activation energy (ΔE_a = slope of Arrhenius plot) is combination of ΔE_a for phosphoryl group transfer (V_m) with the enthalpies (ΔH) of substrate dissociation if the quasi-equilibrium assumption is hold. In addition, the equilibrium ADP concentration could vary due to temperature decrease, if the ΔH of the overall reaction is markedly different from zero (e.g., at $\Delta H = 5$ kcal/mol, a 2-fold change in [ADP] will take place in over a 30 C° temperature interval). However, the ΔH of the reaction is only 0.5 kcal/mol [32] and therefore [ADP] should be nearly constant over the range tested. Obviously, [ATP], [PCr] and [Cr] are in any case constant, as they exceed [ADP] approx. 100-times at 30–37 °C. The contribution of ΔH to ATP and PCr association with the enzyme should be small because of significant saturation of CK with these substrates ($[ATP]/K_m(ATP) = 10$ and $[PCr]/K_m(PCr) = 3.5$) and that of ΔH for Cr binding can be moderate ($[Cr]/K_m(Cr) = 2$). Furthermore, saturation of the enzyme with these substrates becomes higher as temperature decreases due to reduction of K_m values [32], thereby somewhat increasing the fraction of ternary productive complexes. In contrast, the saturation with ADP is considerably less ($[MgADP]/K_m(ADP) = 0.3$). Therefore, enhancement of the MgADP affinity to the enzyme with the temperature drop may partially compensate the decline in the exchange rates induced by the effect of temperature decrease on V_m.

Finally, the stability of magnesium complexes of ATP and ADP increases with temperature fall (approx. 2-times over an 18 C° temperature interval [32]). However, free [Mg^{2+}] in our experiments was about 2.0 mM, providing saturation of ATP and ADP by 95% ($K_a \approx 10^4$ M^{-1}) and 70% ($K_a \approx 10^3$ M^{-1}), respectively. Therefore, MgADP concentration could be only slightly increased due to temperature decrease.

In spite of many factors potentially capable of affecting temperature dependence of creatine kinase-catalyzed exchange rates, apparent ΔE_a values were close to that found for maximal rates (V_m) of this reaction. In fact, E_a values for the exchanges PCr–ATP (NMR), PCr–ATP (isotop.), ATP–ADP (isotop.) and V_m are: 12.0, 13.5, 12.0 and 12.8 kcal/mol, respectively. This suggests that the contribution of the factors listed above to temperature dependence is really small.

Different methods for measurements of exchanges catalyzed by creatine kinase gave similar specific rates over the physiological range of temperature (30–37 °C) and substrate concentration, in spite of the very broad range of enzyme concentrations used. Thus, it was 0.04 mg/ml (1 μM of active sites), 0.4 mg/ml (10 μM) and 2–20 mg/ml (50–500 μM) for [^{3}H]ADP–ATP, [γ-

^{32}P]ATP–PCr exchanges and [γ-P]ATP–PCr magnetization transfer, respectively. Moreover, kinetic constants determined from steady-state kinetics in very diluted enzyme solutions (0.01 μg/ml ≈ 0.25 nM) provided good-fitting calculated rates and measured fluxes at high enzyme concentrations (10 to 500 μM) [5,8]. This may mean that dimeric form of the MM isozyme of creatine kinase remains unchanged in terms of dissociation or oligomerization or that the kinetic properties of these hypothetical forms, if they exist, are not very much different from those of the dimer.

Appendix 1

Theoretical considerations of applying magnetization transfer for determining rates of enzyme-catalyzed reactions

Let us consider the simplest scheme of an enzyme reaction:

$$E+S \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} ES \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} EP \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} E+P$$

Using this scheme we have analyzed application of magnetization transfer for investigation of enzyme reactions. Basically, two cases were considered.

Case 1. Chemical shifts of nuclei of interest in substrate-enzyme complexes (ES and EP) are significantly different from the chemical shifts of the free substrates (S and P) and their lines have sufficiently long T_2 times to resolve individual resonances. This means that it is possible to observe separate signals from the complexes under appropriate conditions (high enough enzyme concentration) and that selective saturation of signals of free substrates can be done. Obviously, the apparent binary exchange S-to-P in this case is a three-step reaction. However, the situation is simplified if the lifetimes, tau, of complexes are much shorter than corresponding T_1 times and the lifetimes of free substrates and, therefore, loss of saturation (or excitation) due to spin-lattice relaxation along the pathway from s to P can be neglected. These conditions are fulfilled when substrate concentration (S or P) to which saturation (or excitation) is being transferred is much higher than that of complexes and also times T_1 for bound substrates are not much shorter than those for free compounds. Thus, a three-step enzymatic reaction (in general, a multistep one) can be treated as single-step reaction with an apparent pseudo-first-order rate constant which can be easily expressed as combinations of elementary rate constants. Obviously, in such a system fluxes measured by the NMR magnetization transfer technique are equal to real fluxes determined, for example, by radioisotope tracer methods.

Case 2. Chemical shifts of the signals of enzyme-bound substrates are close to those of respective free forms and have similar line shapes. This implies that we cannot observe separate peaks of complexes, and S and ES, P and EP, respectively, are indistinguishable in terms of frequency. In this case we can saturate (or excite) only S + ES or P + ES as a single entities. As a consequence, steps of enzyme-substrate complex formation and dissociation are masked and, therefore, measured fluxes are determined mostly by a complex interconversion step (k_{23}, k_{32}). Obviously, these fluxes can be much larger than the actual flux between S and P. If interconversion steps of complexes are rate-limiting, then fluxes observed by magnetization transfer methods may exceed real values only by a relatively small factor. If this is not the case real flux can be overestimated many times.

Another consequence of small chemical shift difference between lines of S and ES, P and EP is that in general 'true' or 'intrinsic' T_1 times determined from magnetization transfer experiments are not true in the sense usually used. In fact, these values refer to S + ES or P + EP and hence depend on several factors such as intrinsic T_1 times for free substrates (S, P) and respective complexes (ES, EP), the ratio of [S]/[ES] or [P]/[EP] (in other words, substrate/enzyme ratio) and the rate constants of formation and breakdown of these complexes. For example, if the intrinsic T_1 of complex (ES or EP) is shorter than the T_1 for free substrates, the corresponding T_1 value detected from magnetization transfer experiments becomes shorter as the enzyme/substrate ratio increases. Therefore, in general, usage of known T_1 times for free substrates to calculate rate constants from steady-state magnetization values obtained at long time saturation can lead to errors. For instance, in the case described above, introduction of intrinsic T_1 for the free substrate (longer than observable) in expression gives underestimated k values, since this formulae is correct for observable T_1.

It is possible to imagine situations intermediate between two of these described above. However, this is out of the scope of this work.

Appendix 2

Calculations of dissociation rate constants of substrate-enzyme complexes from steady-state saturation transfer of invisible lines

Let us consider the binary system:

$$MgADP+E \underset{k_d}{\overset{k_a}{\rightleftharpoons}} E\cdot MgADP$$

where k_a and k_d are association and dissociation rate constants, respectively. When the β-P of E-MgADP is being saturated, the pseudo-first-order rate constant,

k_{obs}, obtained from the dependence of $\ln(M - M_\infty)$ on time is equal to $k_a E$. Obviously, under equilibrium:

$$k_{obs} = \text{flux}/[\text{MgADP}] = k_d[\text{E}\cdot\text{MgADP}]/[\text{MgADP}] \quad (1A)$$

If $[\text{E}\cdot\text{MgADP}] \gg [\text{E}]$, then

$$k_{obs} = k_d E_0/(K_d + [\text{MgADP}]) \quad (2A)$$

where k_d is dissociation constant of the complex. At $[\text{MgADP}] \gg K_d$

$$k_{obs} = K_d E_0/[\text{MgADP}] \quad (3A)$$

Taking into account that the K_m for MgADP ($K_m \cong K_d$) is approximately 0.2 mM and the difference between [MgADP] and E_0 in our experiments is order of several mmol/l, Eqn. 3A is applicable for analysis of our data.

For the case of incomplete saturation of the selected line there is the equation derived by Campbell et al. [29], which for steady-state magnetization (M_z^∞) is:

$$M_z^\infty/M_z^0 = (1/T_1 + fk_1)/(1/T_1 + k_1) \quad (4A)$$

where f is the degree of incompleteness of saturation and $(1 - f)$ is the degree of saturation, respectively.

It is reasonable to transform Eqn. 4A in the following way:

$$1/(1 - M_z^\infty/M_z^0) = 1/(1-f) + 1/Tk(1-f) \quad (5A)$$

Introducing expression 3A for k into Eqn. 5A we obtain:

$$1/(1 - M_z^\infty/M_z^0) = 1/(1-f) + [k_d T_1(1-f)]^{-1} \times [\text{MgADP}]/[\text{E}]_0 \quad (6A)$$

Obviously, this is equation of the straight line of $1/(1 - M_{z\infty}/M_{z0})$ vs. [MgADP]/[E], the intercept of which with the abscissa is $-1/k_d$ and does not contain the saturation factor, $(1 - f)$. If T_1 for MgADP is known, the dissociation constant of E · MgADP can be calculated by plotting $1/(1 - M_z^\infty/M_z^0)$ as a function of the [MgADP]/[CK] ratio when the β-P line of E · MgADP is saturated.

References

1 Morrison, J.F. and Cleland, W.W. (1966) J. Biol. Chem. 241, 673–683.
2 Morrison, J.F. and James, E. (1965) Biochem. J. 97, 37–52.
3 Brindle, K.M., Porteus, R. and Radda, G.K. (1984) Biochim. Biophys. Acta 786, 18–24.
4 Kantor, H.L., Ferretti, J.A. and Balaban, R.S. (1984) Biochim. Biophys. Acta 789, 128–135.
5 Kupriyanov, V.V., Steinschneider, A.Ya., Ruuge, E.K., Kapelko, V.I., Zueva, M.Yu., Lakomkin, V.L., Smirnov, V.N. and Saks, V.A. (1984) Biochim. Biophys. Acta 805, 319–331.
6 Brindle, K. and Radda, G.K. (1985) Biochim. Biophys. Acta 829, 188–201.
7 Degani, H., Laughlin, M., Campbell, S. and Shulman, R.G. (1985) Biochemistry 24, 5510–5517.
8 Kupriyanov, V.V., Lyulina, N.V., Steinschneider, A.Ya., Zueva, M.Yu. and Saks, V.A. (1986) FEBS Lett. 208, 89–93.
9 Kupriyanov, V.V., Balaban, R.S., Steinschneider, A.Ya., Lakomkin, V.L., Ruuge, E.K., Smirnov, V.N. and Saks, V.A. (1987) in Myocardial Metabolism. Cardiology Reviews, Vol. 2 (Smirnov, V.N. and Katz, A.M., eds.), pp. 297–325, Harwood, Switzerland.
10 Brown, T.R., Gadian, D.G., Garlic, P.B., Radda, G.K., Seely, J.P. and Styles, P. (1978) Frontiers in Biological Energetics, Vol. 2, 1341–1349, Academic Press, New York.
11 Nunnaly, R.L. and Hollis, D.P. (1979) Biochemistry 18, 3642–3646.
12 Matthews, P.M., Bland, J.L. and Radda, G.K. (1982) Biochim. Biophys. Acta 721, 312–320.
13 Bittl, J.A. and Ingwall, J.S. (1985) J. Biol. Chem. 260, 3512–3518.
14 Koretsky, A.P., Wang, S., Klein, M.P., James, T.L. and Weiner, M.W. (1986) Biochemistry 25, 77–84.
15 Ugurbil, K., Petein, M., Maidan, R., Michurski, S. and From, A.H.L. (1986) Biochemistry 25, 100–107.
16 Bendall, R., Cartwright, I.L., Clark, P.I., Lowe, G. and Nurse, D. (1977) Eur. J. Biochem. 79, 201–209.
17 Cayley, P.J., Albrand, J.P., Feeney, J., Roberts, G.C.K., Piper, E.A. and Burgen, A.S.V. (1979) Biochemistry 18, 3886–3895.
18 Hyde, E.I., Bridsall, B., Roberts, G.C.K., Feeney, J. and Burgen, A.S.V. (1980) Biochemistry 19, 3738–3746.
19 Forsen, S. and Hoffman, R.A. (1963) J. Chem. Phys. 39, 2892–2901.
20 Morris, J.A. and Freeman, R. (1978) J. Magn. Res. 29, 397–417.
21 Carr, H.Y. and Purcell, E.M. (1954) Phys. Rev. 94, 630–638.
22 Meiboom, S. and Gill, D. (1958) Rev. Sci. Instrum. 29, 688–691.
23 Savabi, F., Geiger, P.J. and Bessman, S.P. (1984) Anal. Biochem. 137, 374–379.
24 Keutel, H.J., Jacobs, H.K., Ziter, F., Mahland, L. and Kuby, S.A. (1972) Arch. Biochem. Biophys. 150, 648–678.
25 Annesley, T.M. and Walker, J.B. (1977) Biochim. Biophys. Res. Commun. 74, 185–190.
26 Kupriyanov, V.V., Panasenko, N.A. and Saks, V.A. (1980) Bioorganicheskaya Khimiya 6, 116–137 (in Russian).
27 Rao, B.D.N. and Cohn, M. (1981) J. Biol. Chem. 256, 1716–1721.
28 Watts, D.C. (1971) in The Enzymes, 2nd Edn. (Boyer, P.D., ed.), Vol. 8, pp. 383–455, Academic Press, New York.
29 Travers, F., Barman, T.E. and Bertrand, R. (1979) Eur. J. Biochem. 100, 149–155.
30 Barman, T.E., Brun, A. and Travers, F. (1980) Eur. J. Biochem. 110, 397–403.
31 Campbell, I.D., Dobson, C.M., Ratcliffe, R.G. and Williams, R.J.P. (1978) J. Magn. Res. 29, 397–417.
32 Kuby, S.A. and Noltmann, E.A. (1962) in The Enzymes, 2nd Edn. (Boyer, P.D., ed.), Vol. 6, pp. 515–597, Academic Press, New York.
33 Kupriyanov, V.V., Steinschneider, A.Ya., Ruuge, E.K., Smirnov, V.N. and Saks, V.A. (1983) Biochem. Biophys. Res. Commun. 114, 117–125.