EFFECT OF pH AND INORGANIC PHOSPHATE ON CREATINE KINASE INACTIVATION: AN IN VITRO $^{31}$P NMR SATURATION-TRANSFER STUDY

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SUMMARY: The pseudo-first-order rate constant of rabbit muscle creatine kinase (CK), in the direction of ATP synthesis ($k_f$), was determined by saturation-transfer $^{31}$P NMR. When pH was varied between 6.0 and 7.4, $k_f$ increased linearly at both 20°C and 37°C. The corresponding flux is very small between pH 6.0 and 6.5, in contrast to previous studies. Up to 50 h exposure of the CK enzyme to high concentrations of inorganic phosphate ($P_i$), a known inhibitor in certain situations, had negligible effect on enzymatic flux in the physiological pH range. Thus under in vivo conditions, such as in stroke, where pH falls as low as 6.2 and $P_i$ rises to high levels, the rate of the CK reaction may be severely reduced due to pH but not due to high $P_i$ concentrations.

INTRODUCTION

Creatine kinase (CK, EC 2.7.3.2) catalyzes the synthesis of adenosine triphosphate (ATP) from phosphocreatine (PCr) and adenosine diphosphate (ADP) in the presence of Mg$^{++}$ and H$^+$. 

$$k_f, k_r$$

$$\text{PCr}^{2-} + \text{MgADP}^{2-} + H^+ \overset{k_f, k_r}{\longrightarrow} \text{Creatine} + \text{MgATP}^{2-}$$ (1) 

CK

Numerous studies of reaction rates, aimed at explaining the regulatory energy buffer role of CK, have been performed in vitro (1-3) and in vivo (4-8) using $^{31}$P saturation-transfer NMR. Saturation-transfer has advantages over biochemical methods since it provides a direct measure of phosphoryl group transfer in real time, without use of labels, and allows for simultaneous measurement of energy metabolites and pH. As such, the reaction can be monitored for extended periods of time.
Inorganic phosphate (P\textsubscript{i}) is known to be a competitive inhibitor with respect to PCR in the forward direction and may also alter protection of an inactive Cr-E-MgADP complex by certain planar anions (9,10). In addition, P\textsubscript{i} has been shown to have a number of effects on mitochondrial CK. The presence of 2-25 mM P\textsubscript{i} has a marked influence on the self-aggregation of the enzyme (11,12). Exposure to 25 mM P\textsubscript{i} for periods of several days results in enzyme precipitation (12). Following severe ischemia or human stroke the infarcted region of the brain may become exposed to concentrations of inorganic phosphate exceeding 20 mM in addition to pH as low as 6.2 for 36 h (13,14). Although the effect of pH on K\textsubscript{eq} is carefully documented (15), discrepancies still exist in quantitation of the influence of pH on the forward CK flux (1,10,16,17). This might be crucial in explaining the energy failure which occurs during stroke.

Using purified rabbit muscle CK we have examined the effects of P\textsubscript{i} and pH on enzymatic flux in the direction of ATP synthesis by the NMR saturation-transfer method under conditions similar to that found in human stroke.

MATERIALS AND METHODS

Rabbit muscle CK (Type I, Sigma) with a specified activity of 185 units/mg was used throughout. Solutions contained 160 μM CK, 5.0 mM PCR, 3.5 mM ATP, 3.9 mM MgCl\textsubscript{2}, 5.0 mM creatine, and 200 mM PIPES buffer. To minimize microbe growth, 0.01 mM EDTA was added to the filtered solutions, and sterilized tubes were used throughout. Temperature was maintained at either 20 or 37 °C for the pH measurements. The pH was measured both before and after placing each sample tube into the NMR magnet and was checked during the NMR experiment from the chemical shift difference between P\textsubscript{i} and PCR. The difference was compared with a standard literature pH calibration curves (18).

\textsuperscript{31}P NMR experiments were performed at 162.0 MHz with a Bruker AM-400 WB spectrometer. Magnetic field homogeneity was optimized on the water proton signal from each sample so that the subsequent \textsuperscript{31}P signals were less than 10 Hz in linewidth (Figure 1). Saturation-transfer was performed using a DANTE selective irradiation experiment (19) as applied in previous studies (20,21). At 20°C DANTE saturation of the γ-phosphate resonance of ATP (γ-ATP) was obtained using a 10 s train of 40,000 individual 0.6 us pulses, each separated by a 250 us delay. Saturation of the NMR signal was better than 98% effective, thus permitting accurate measurement of phosphoryl transfer from phosphocreatine. At 37°C, the saturation time was increased to
15 s using 60,000 pulses in order to compensate for the longer relaxation time of PCR. The steady-state saturation was briefly interrupted by a non-selective 12 us (90°) pulse and 84 ms of acquisition time. The sequence was accumulated for 64 repetitions and followed by an identical control experiment except the DANTE pulse was applied at a frequency off-resonance from the PCR peak by a distance equal to the separation between γ-ATP and PCR. This control procedure compensates for non-specific, direct saturation effects. Following Fourier transformation, the enzymatic pseudo-first-order rate constant (k_f) can be directly calculated by the equation (22-25):

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k_f = (T_{1m}^{-1})(1-M^+/M_o)
\]  

(2)

where M^+ is the PCR amplitude obtained while γ-ATP was saturated and M_o is the PCR amplitude from the control spectrum. T_{1m} is the spin-lattice relaxation time (T_1) of PCR measured during saturation of γ-ATP. T_{1m} was measured in a separate experiment by the inversion-recovery method (26) and analyzed by a 3-parameter fit (27,28). Since the ratio of T_{1m} to T_1 is equal to M^+/M_o, calculations of the spin-lattice relaxation times are simplified (24). Flux was determined from the product of the rate constant and the PCR concentration for this first order reaction (3). The initial PCR concentration of 5.0 mM was corrected, by the loss due to hydrolysis, using NMR peak areas of PCR and P_i. ATP was quantitated from the area of the β-ATP peak.

RESULTS

NMR measurements of the fraction of transfer of magnetization from the PCR resonance position have been made during irradiation of the γ-phosphate resonance of ATP at 20°C and 37°C as a function of time and pH in solutions containing minimal P_i (less than 1 mM). The CK concentration of 160 μM (13 mg/ml) was chosen in order to increase the measured first-order rate constant, thus providing statistically significant changes in the PCR reduction ratio, M^+/M_o. Figure 1 demonstrates a 60% reduction in the PCR amplitude when saturating γ-ATP in the presence of CK at 37°C, pH 7.27. The γ-ATP peak appears completely saturated in Figure 1b. Using a T_{1m} value of 1.7 s, k_f is calculated to be 0.35 s^{-1} from Equation (2).

At 37°C PCR hydrolysis resulted in significant P_i concentration (>3 mM) after 6-7 h. At 20°C hydrolysis was small as the PCR concentration decreased by less than 10% after 1 day. Therefore, the serial profile of k_f was determined at 20°C in the physiological pH range and the results at pH 6.45 and 7.15 are
Figure 1. $^{31}$P NMR spectra used for the calculation of magnetization transfer by CPK in the direction of ATP synthesis. The sample was prepared in PIPES buffer and maintained at 37°C and pH 7.27. Peak assignments are (1) P$_i$, (2) PCR, (3) γ-ATP, (4) α-ATP, (5) β-ATP. Arrows denote the selective saturation of the γ-ATP resonance in (B) and at an equal frequency to the left of PCR in the control spectrum (A). The difference spectrum (A-B) is shown in (C). The NMR parameters are described in the Methods section.

illustrated in Figure 2. The $k_f$ remained nearly constant at 0.07 s$^{-1}$ and 0.2 s$^{-1}$ respectively during the first 16 h of reaction. At that time, acquisition was briefly interrupted and inorganic phosphate was added to the NMR tube for a final concentration of either 17 or 25 mM. As seen from Figure 2, there is no decrease (small positive slope) in the $k_f$ values obtained during the next day at either solution pH. Since PCR was nearly constant at 20°C, the flux in the forward direction was also constant at 0.35 mM/s and 1.0 mM/s respectively.

Although there was no significant change in enzyme flux at high concentrations of P$_i$, flux was noted to drop by three fold
Figure 2. Serial profile of $k_f$ for the creatine kinase reaction. The upper curve was obtained at pH 7.15 while the lower was at pH 6.45-6.50. The open symbols represent rates measured subsequent to addition of 17 or 25 mM $P_i$. Each of the two samples remained in the magnet for data acquisition for nearly two days. The uncertainty in $k_f$ was about 0.01 s$^{-1}$ determined from repeat measurements of the PCR reduction ratio $M^+/M_0$.

with a 0.7 unit decrease in pH. In fact, at constant temperature and constant excess total magnesium, a decrease in pH almost linearly inhibits the action of the enzyme until it has very limited activity near pH 6.0. This is clearly shown at both 20°C and 37°C in Figure 3, where each data point represents an average of two independent measurements. Separate enzyme solutions were prepared from frozen stock solutions for each pH value to insure for consistent experiments and minimal hydrolysis. The slope of the plot of rate constant with pH is 0.16 +/- 0.009 s$^{-1}$ per pH unit ($r^2=.97$) at 20°C and 0.24 +/- 0.007 s$^{-1}$ per pH unit ($r^2=.99$) at 37°C. In additional experiments, the concentration of $P_i$ was raised to 25 mM but had little effect on $k_f$ over a pH range of 5.8 - 7.4. We have chosen to examine the muscle form of creatine kinase due to its high purity and low cost. Differences in rates measured with brain isoenzyme were not evaluated in this preliminary study.
Figure 3. Pseudo-first-order rate constant in the direction of ATP synthesis as a function of pH at 20°C and 37°C. Each point represents the mean of 2 measurements which were completed within 1 h after sample preparation. T, in the absence of exchange was 2.03 s at 20°C and 4.34 s at 37°C. Equation (2) was used in calculating $k_f$.

DISCUSSION

NMR magnetization-transfer methods are now widely used for measuring enzyme-catalyzed fluxes. Using this method, the forward flux of the CK reaction in healthy rat brain was estimated to be 0.26 s$^{-1}$ (1.6 umol/s per g wet wt) with a smaller value for the ATP synthetase reaction (4). The NMR technique avoids difficulties associated with coupled enzyme system determinations of CK activity (17) but makes the assumption of a two-site exchange in deriving Equation (2) (2,22-25). Since most investigations performed on living tissues concerning CK fluxes now use NMR magnetization-transfer (4-8), it should be preferable that control experiments use similar methodology. In the in vitro study reported here, ATP concentrations did not change significantly with time at either 20°C or 37°C. Therefore, the steady-state conditions of the concentrations of phosphorous metabolites required for saturation-transfer experiments were satisfied.
In previous studies, the pH optima of the activity of muscle creatine kinase in the direction of ATP synthesis was determined to be between 6.0 and 7.0 (16) or below 6.0 (10). The choice of buffers is likely a source for the differences in these early studies. The availability of Mg$^{2+}$ for ATP may vary significantly since it binds with different affinities to various buffers. Szasz et al., using several buffers including PIPES determined a broad pH optimum at 6.7 with significant activity at pH 6.0 (17). However in vivo saturation-transfer NMR measurements performed at pH 6.5 and 7.4 gave a 40% greater flux at the higher pH (1). This result is somewhat in agreement with our measured increase in flux of over 100% between these values (Fig. 1).

The present investigation indicates that the transfer of saturation or the flux in the direction of ATP synthesis is almost negligible at pH 6.0. These results would support a model of less than one-third the ATP synthesis by CK at pH 6.2 compared with the intracellular pH of 7.1 in healthy brain (13,14). Thus, the usefulness of PCR as an energy reserve may be severely compromised by the pH found during the first 36 h in post cerebral infarct or after excessive excercise. Secondly, in vitro exposure to high concentrations of P$_i$ for prolonged periods (2 days) had minimal effect on enzymatic flux. Our results suggest that P$_i$ does not alter muscle CK by promoting aggregation or by stabilization of an inactive intermediate complex such as Cr-E-MgADP.

REFERENCES