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Compartmentation of inorganic phosphate in perfused rat liver

Can cytosol be distinguished from mitochondria by ^{31}P NMR?

Eric Thiaudiere*, Jean-Louis Gallis, Sylvie Dufour, Nicole Rousse, Paul Canioni

Laboratoire de Résonance Magnétique cellulaire et de régulation des flux métaboliques, IBC-CNRS, 1, rue Camille Saint-Saens, F-33077 Bordeaux, France

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Compartmentation of inorganic phosphate was studied in intact perfused rat liver at 4°C by ^{31}P NMR. It was shown that decreases in cytosolic pH or cytosolic P_i concentration induced the appearance of an additional P_i resonance at low field which was assigned to P_i from an alkaline compartment. Valinomycin (K^+ ionophore) induced a further splitting of the lines whereas nigericin (K^+/H^+ antiport) or potassium cyanide (inhibitor of cytochrome oxidase) had opposite effects. As valinomycin acts mainly on the cytosolic/mitochondrial K^+ gradient and KCN on the mitochondrial respiratory chain, it was deduced that the alkaline compartment as revealed by the second P_i resonance was probably mitochondria. Single P_i lines observed on perchloric extracts of livers exhibiting two resonances during cold perfusion confirmed that the split peaks in the intact liver indeed arose from the same molecular species.

Compartmentation; pH; Cytosol; Mitochondria; Magnetic resonance; Rat liver

1. INTRODUCTION

Studies on hepatic metabolism by phosphorus-31 nuclear magnetic resonance started in 1978 on isolated rat liver cells [1] The technique was then successfully applied for investigations on the whole perfused organ from rat [2] or mouse [3]. ^{31}P NMR of perfused livers gives information not only on the energy status of the organ from the well resolved signals of high energy metabolites as nucleoside triphosphates (NTP), but also on the intracellular pH, since the chemical shift of the inorganic phosphate resonance (P_i) varies according to its protonation state [4]. The existence of a well resolved P_i resonance has been reported on isolated mitochondria [5], which means that, in cells or organs, one should expect to separate the resonance of the cytosolic P_i from the mitochondrial one if their respective pH are different. Desmoulin et al. [6] claimed that this distinction is not possible in rat livers perfused at 37 °C with valinomycin, a K^+ ionophore which is supposed to increase the mitochondrial/cytosolic pH gradient [7]. On the other hand, decreasing the temperature to 4°C induced a sharpening of the signals in the P_i spectral region, which increased the apparent spectral resolution [8]. Indeed, mitochondria could be discriminated from cytosol on isolated hepatocytes suspended in the NMR cell at 4°C [1]. In the present study, we investigated the behaviour of ^{31}P NMR signals in the P_i spectral region of isolated rat livers perfused at 4°C. First, by decreasing the pH of the perfusion medium, we showed that the cytosolic P_i peak can be shifted, making possible the observation of another signal in the P_i resonance region. The use of K^+ ionophore as valinomycin which acts essentially on the mitochondrial membranes, KCN, inhibiting respiration, and nigericin (K^+/H^+ antiport), brought strong arguments that this additional inorganic phosphate resonance could be attributed to the inorganic phosphate of the mitochondrial compartment.

*Corresponding author. Fax: (33) 56 98 24 33.

Abbreviations: DPDE, diphospho-diesters GPC, glycerophosphocholine; GPE, glycerophospho-ethanolamine; KHB, Krebs–Henseleit buffer; MDPA, methylenediphosphonic acid; NAD, nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; NDP, nucleoside-5′-diphosphates; NTP, nucleoside-5′-triphosphates; PEP, phosphoenolpyruvate; pH_e, extracellular pH; pH_i, intracellular pH; P_i, inorganic phosphate; PME, phosphomonoesters; UDPG, uridyldiphosphoglucose.

2. EXPERIMENTAL

2.1. *Liver perfusion*

Male Wistar rats (80–120 g) were fed ad libitum and anesthetized by intraperitoneal injection of pentobarbital sodium (100 mg/kg). Liver (4–6 g) was exposed and the portal vein was immediately cannulated with a teflon cannula through which the perfusion medium at 4°C was perfused at 2 ml/min/g wet weight. The liver was then removed from the animal and introduced in a 20 mm diameter NMR cell. The perfusion system was already described [8]. O_2 consumption of the liver was assessed by O_2 Clark electrodes, placed in the perfusion circuit. The initial perfusion medium was a Krebs–Henseleit buffer (KHB) in which the composition was (in mM): NaCl 119, $NaHCO_3$ 25, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.3, pH 7.4. In this case the medium was gassed through a membrane oxygenator [4] with a 95% O_2/5% CO_2 mixture. When necessary, the concentration of

HCO_3^- or inorganic phosphate was modified, or pH decreased with HCl or by modifying the K_2HPO_4/KH_2PO_4 ratio. When HCO_3^- was removed, the gas used was pure O_2.

2.2. *NMR spectroscopy*

^{31}P NMR spectra of liver were recorded without proton decoupling on a Bruker AM400 spectrometer operating at 9.39 Tesla (161.9 MHz). 240 free-induction decays (47.5° flip angle, 0.2048 s aquisition time, 0.5 s delay between pulses, 4 Ko data points) were routinely acquired within 2 min, 15 Hz line-broadened, and fast Fourier-transformed by the Bruker software.

^1H-decoupled ^{31}P-NMR spectra of perchloric acid extracts of livers (see below) were run in 5 mm NMR cells at 20°C. Proton decoupling was performed by a WALTZ 16 sequence applied during the aquisition period. Typically 36,000 free inductions decays (30° flip angle, 0.4096 s aquisition time, 0.5 s delay between pulses, 8 Ko data points) were recorded and Fourier-transformed after 3 Hz line-broadening.

Peak assignment was done as previously described [4] using methylene diphosphonic acid (MDPA) as an external standard. Its chemical shift was set at 18.4 ppm.

pH determination from the chemical shift of P_i at 4°C was done according to appropriate titration curves which had been drawn using a buffer reflecting the intracellular ion composition [4].

2.3. *Liver extracts*

After the NMR experiment, livers were freeze-clamped with aluminium tongs precooled in liquid nitrogen. Extraction of metabolites was carried out with 7 M perchloric acid, subsequently neutralized by potassium hydroxide, as already described [6]. Extracts were then freeze-dried and resuspended in D_2O containing 50 mM EDTA. pH of extracts were measured within the NMR cell.

3. RESULTS

A typical NMR spectrum of a liver perfused at 4°C with KHB for an external pH (pH_e) of 7.4 is shown in Fig. 1a. It exhibits only one resonance in the 1–3 ppm range corresponding to P_i. Due to its very long (10 s) spin-lattice relaxation time (T_1), the external P_i contributes only for less than 5% to this line which thus reflects the intracellular P_i (T_1 value for cytosolic P_i was around 0.8 s at 4°C [4]). From the chemical shift value (2.62 ppm), one can estimate an intracellular pH_i of 7.32. For the whole set of studied livers at pH_e 7.2, we obtained a mean value of 7.24 ± 0.07 (S.D., $n = 8$). Liver perfused without HCO_3^-, at an external pH_e of 7.2, resulted in spectra as depicted in Fig. 1b. Two resonances of P_i were observed at 2.87 ppm (pH 7.62) and 2.52 ppm (pH 7.22), indicative of the presence of two compartments. For all the data, we observed pH_1 7.11 ± 0.05, ($n = 11$, $P < 0.001$), and pH_2 7.47 ± 0.11, ($n = 7$) at pH_e 7.2. In order to discriminate the intrinsic effect of pH_e from [HCO_3^-] on the P_i resonance splitting, we performed experiments with HCO_3^--free media at different pH_e. Starting the perfusion at pH 7.2 (Fig. 1b), a very clear separation occurred by an acute change in pH_e down to a value of 6.7 (Fig. 1c). The two pH_i values were then 7.62 and 6.61. The presence of large amounts of a phosphorylated compound must be noticed that resonates at −0.5 ppm, and which had been attributed to PEP [9].

In order to assess the origin of this additional

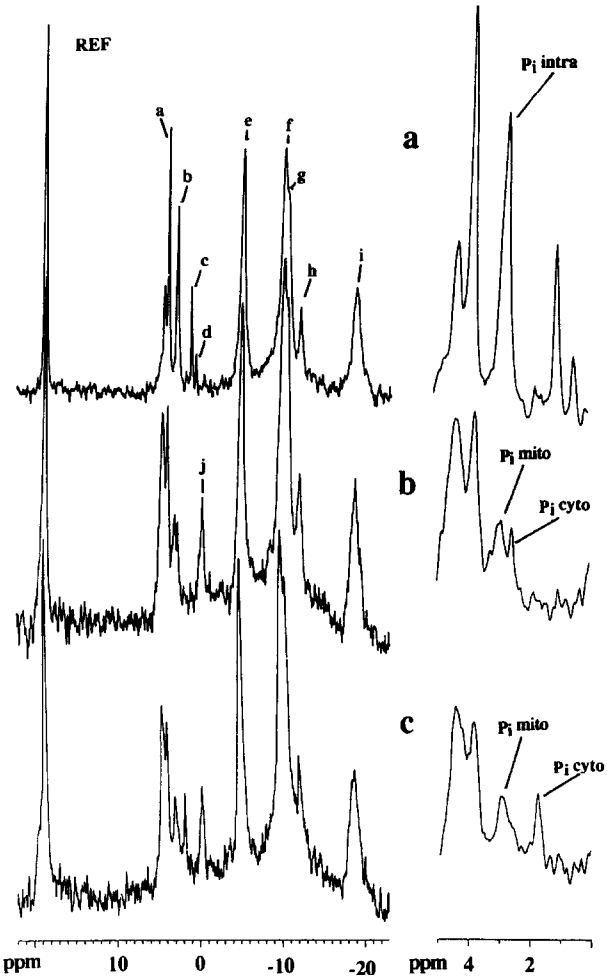

Fig. 1. Role of HCO_3^- and external pH on the ^{31}P NMR resonance of P_i in perfused rat liver at 4°C. (**a**) Perfusion with KHB in the presence of 25 mM HCO_3^-, pH_e 7.4. Lines were attributed according to Delmas-Beauvieux et al. [4]. Transplantation: (Ref) methylenediphosphonic acid (MDPA), taken as chemical shift reference at 18.4 ppm; (a) phosphomonoesters (PME), (b) inorganic phosphate (P_i); (c) glycerophosphoethanolamine (GPE); (d) glycerophosphocholine (GPC); (e) nucleoside-5′-triphosphates (NTP_g) and nucleoside-5′-diphosphates (NDP_b); (f) NTPa and NDP_a; (g) nicotinamide adenine dinucleotide (NAD and NADH); (h) uridyldiphosphoglucose (UDPG); (i) NTP_b; P_i^{intra}, intracellular P_i. (**b**) Perfusion medium without HCO_3^-/CO_2, pH 7.2; (j) phosphoenolpyruvate (PEP); P_i^{mito}, mitochondrial P_i; P_i^{cyto}, cytosolic P_i. (**c**) Same liver perfused without HCO_3^-/CO_2, pH 6.7. The 0–5 ppm region is expanded for each spectrum.

phosphate resonance, we chose to decrease the external concentration of P_i down to 40 μM instead of 1.2 mM, in the presence of 25 mM HCO_3^-, pH 7.4. Under such conditions, partial depletion of the cytosolic compartment in P_i was expected, and thus reveal the other resonance. As shown in Fig. 2a, a shoulder rose at low field. After addition of 50 nM valinomycin, a potassium ionophore binding to mitochondrial membranes, a net splitting appeared between the two peaks, and the alkaline component rose up (Fig. 2b). pH values were 7.49 ± 0.08

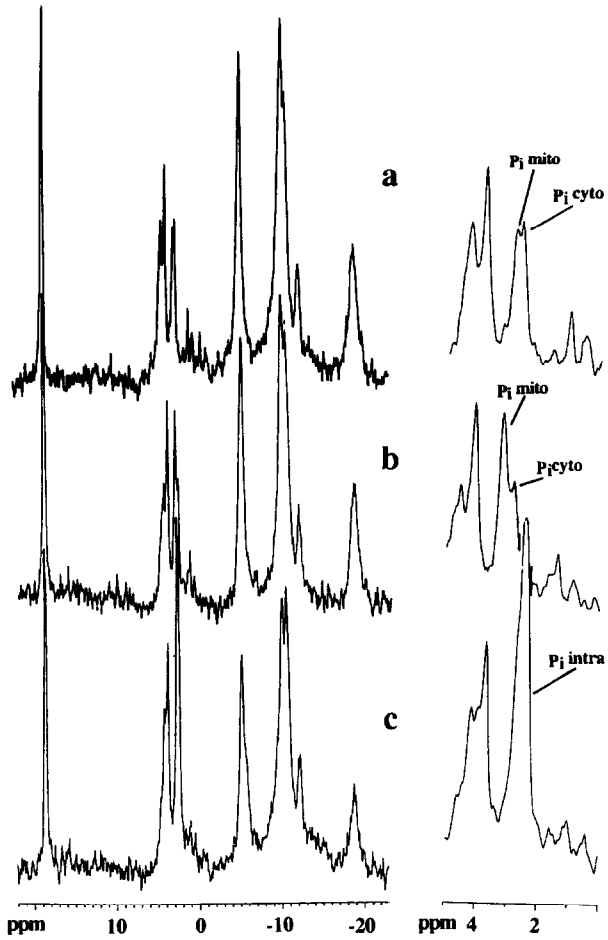

Fig. 2. Effect of decreasing concentration of P_i, and of valinomycin and nigericin addition on the splitting of P_i resonances in perfused rat liver at 4°C. (a) KHB with 25 mM HCO_3^- and 40 mM P_i, pH_e 7.4. (b) The same with 50 nM valinomycin. (c) As in (b) with 60 nM nigericin.

and 7.14 ± 0.05 (n = 4). The kinetics of the splitting of P_i resonances for one experiment are shown in Fig. 3. After 30 min valinomycin treatment, the pH gradient between compartments increased from 0.16 ± 0.04 to 0.34 ± 0.04. A transient relative increase in O_2 consumption of about 4% was observed at the beginning of valinomycin perfusion. The effect of 60 nM nigericin, a K^+/H^+ antiport [9] is depicted in Fig. 2c. The two P_i resonances collapsed perfectly, and a single pH value was determined as 7,06 ± 0.12, (n = 2). In other experiments, perfusion with 2.5 mM KCN (inhibitor of mitochondrial cytochrome oxidase) during the presence of valinomycin was also followed by a collapse of the lines (not shown).

Livers exhibiting two resonances in the P_i region were freeze-clamped, and perchloric acid extractions were performed. ^{1}H-decoupled ^{31}P NMR spectra of such extracts recorded at high spectral resolution exhibited only one P_i peak (Fig. 4), indicating that both lines observed within the 1–3 ppm range in the intact liver are indeed due to P_i present in two different compartments displaying two different pH values.

4. DISCUSSION

In a previous work, Desmoulin et al. [6] did not observe any P_i compartmentation in rat livers perfused at 37°C, even in the presence of 50 nM valinomycin. Here, we show that the role of hypothermia to detect more than one P_i resonance is of critical importance. Changing the temperature from 37 to 4°C induced a very strong line-sharpening effect in the 0–5 ppm region in the intact liver [4,8]. This effect is specific for the intracellular P_i, because it was not found in perchloric extracts, suggesting a special relaxation process in a living environment.

Valinomycin and KCN effects strongly indicate that the additional alcaline compartment is made up by mitochondria. Indeed, the perturbation induced by valinomycin on the K^+ distribution across the mitochondrial membrane would likely provoke an expense of energy to counterbalance it, and then an extrusion of proton

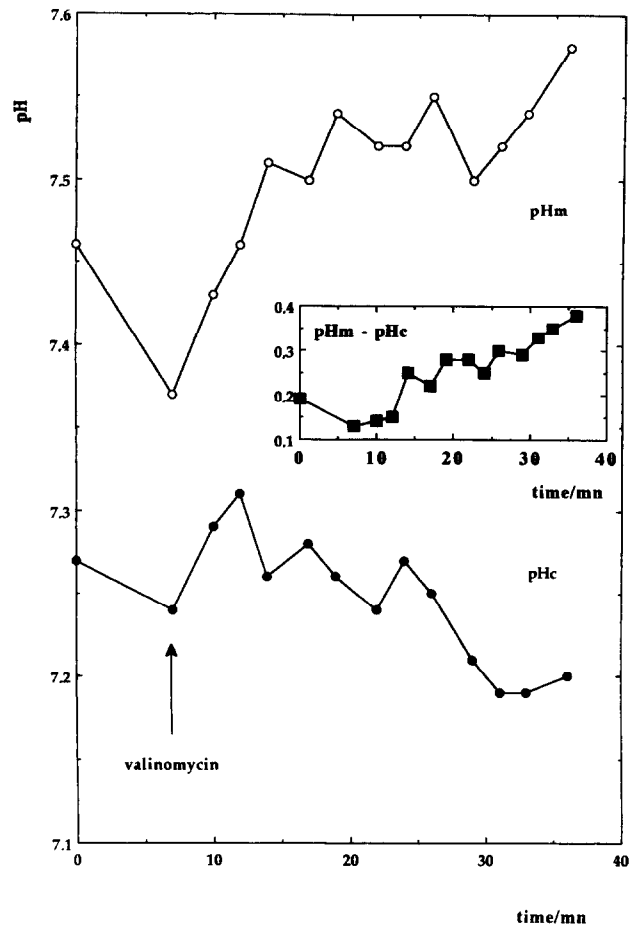

Fig. 3. Time course of pH of compartments in a perfused rat liver at 4°C in the presence of 50 nM valinomycin (insert: pH gradient between mitochondria and cytosol). pH_m, mitochondrial pH; pH_c, cytosolic pH. Perfusion medium: KHB with 25 mm HCO_3^-, pH 7.4.

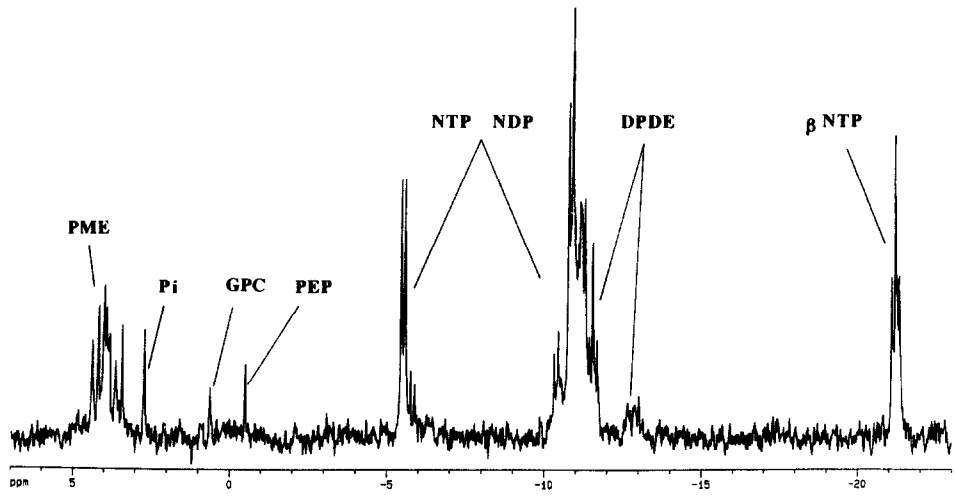

Fig. 4. ^{1}H-decoupled ^{31}P NMR spectrum of a perchloric extract of a liver perfused with KHB in the absence of HCO_3^-/CO_2, pH_e 6.7. Peak assignments was done according to [6]. DPDE, diphospho-diesters.

in the cytosol thanks to respiration. This is somehow correlated by the small increase in O_2 consumption observed at the begining of valinomycin perfusion. When inhibiting respiration upon KCN addition, one could then expect an opposite effect and thus a collapse of the two P_i resonances. This is indeed what we observed. Even though nigericin at 10 mM acts mainly on the H^+/K^+ distribution across the plasma membrane [10], we can assume that a low concentration such as 60 nM should interact with mitochondrial membranes owing to the large surface-to-volume ratio of those organelles and thus might cancel the effect of valinomycin. This is likely the reason why a collapse of the two P_i resonances occured during nigericin perfusion. Our results were in agreement with the observation of two pools of inorganic phosphate on isolated rat hepatocytes using the same technique [1]. However, our data showed that the use of such ionophores is not absolutely necessary to distinguish both cytosolic and mitochondrial P_i. In turn, decreasing cytosolic pH under systemic acidosis (pH_e 6.7) in the absence of HCO_3^- is sufficient to induce the desired splitting of the P_i lines.

The possibility must also be considered of two different types of cells exhibiting distinct reactivities towards valinomycin and nigericin, thus inducing the appearance of two P_i resonances. This hypothesis has been ruled out simply by removing both Na^+ and HCO_3^- in our perfusion media at pH_e 6.7, preventing then the cytosolic pH regulation by Na^+:HCO_3^- symport and Na^+/H^+ antiport. The splitting between both P_i resonances was the same as when Na^+ was present (ΔpH 1.07 ± 0,1, n = 9, not shown). So, if the compartment exhibiting a pH of 7.5 under systemic acidosis (pH_e 6.7) was cytosol, this would imply that H^+ consumption inducing a $\Delta pH_{(i-e)}$ of 1.0 would occur via the intermediate metabolism, which seems very unlikely.

The existence of such large differences in pH between cytosol and mitochondria in the absence of HCO_3^- has to be questioned. Mitochondrial respiration alone would hardly explain them, because we did not observe a large stimulation of oxygen consumption during the transition from pH_e 7.2 to pH_e 6.7. Moreover, as we observed rather large amounts of PEP under such conditions (Fig. 1b and c), it is possible to evoke a higher rate of glycolysis and then an energy supply to maintain the pH gradient. Thus, liver cells would preserve alkaline pH in the mitochondria whatever the cytosolic pH values. Experiments are in progress to investigate the mitochondrial pH regulation in intact perfused rat liver at 4°C.

Thoma and Ugurbil [11] also tried to investigate the compartmentation on isolated rat liver using ^{31}P NMR of P_i and fructose-1-phosphate and ^{19}F NMR of difluoromethylalanine: they found under standard perfusion conditions (37°C and 25 mM bicarbonate) two compartments exhibiting two different pH_i, 7.0 and 7.4. Although the data presented in their paper were difficult to interpret, strong arguments were given in favor of the discrimination between cytosolic and mitochondrial compartment. Recently, Cohen, by using ^{31}P NMR on mouse livers at 12 °C also showed two P_i resonances using 15 nM valinomycin and thus concluded to the appearance of the mitochondrial compartment [12].

In conclusion, this study shows that a new insight has been gained in the field of intact liver metabolism. The discrimination between cytosol and mitochondria in perfused rat liver should allow the careful study of pH regulation in both compartments by ^{31}P NMR. Another application would be the study of mitochondrial integrity under drastic conditions like anoxia or ischemia, which should greatly help the understanding of organ preservation.

REFERENCES

[1] Cohen, S.M., Ogawa, S., Rottenberg, H., Glynn, P., Yamane, T., Brown, T.R. and Shulman, R.G. (1978) Nature 273, 554–556.
[2] Salhany, J.M., Stohs, S.E.J., Reinke, L.A., Pieper, J.M. and Hassing, J.M. (1979) Biochem. Biophys. Res. Commun. 86, 1077–1083.
[3] McLaughlin, A.C., Takeda, H. and Chance, B. (1979) Proc. Natl. Acad. Sci. USA 76, 5445–5449.
[4] Delmas-Deauvieux, M.C., Gallis, J.L., Rousse, N., Clerc M. and canioni, P. (1992) J. Hepatol. 15, 192–201.
[5] Ogawa, S., Rottenberg, H., Brown, T.R., Shulman, R.G., Castillo, C.L. and Glynn, P. (1978) Proc. Natl. Acad. Sci. USA 75, 1796–1800.
[6] Desmoulin, F., Canioni, P., Crotte, C., Gérolami, A. and Cozzone, P.J. (1987) Hepatology 7, 315–323.
[7] Padan, E.. and Rottenberg, H. (1973) Eur. J. Biochem. 40, 431–437.
[8] Gallis, J.L., Delmas-Beauvieux, M.C., biran, M., Rousse, N., Durand, T. and Canioni, P. (1991) NMR in Biomedicine, 4, 279–285.
[9] Matsui, K. and Kojima, M. (1991) Biochem. Int. 24, 1025–1031.
[10] Thomas, J.A., Buchsbaum, R.N., Zimaniak, A. and Racker, E. (1979) Biochemistry 19, 2210–2218.
[11] Thoma, W.J. and Ugurbil, K. (1988) NMR in Biomedicine 1, 95–100.
[12] Cohen, S.M. (1992) SMRM 11th annual meeting, p. 486.