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The B isozyme of creatine kinase is active as a fusion protein in *Escherichia coli:* in vivo detection by ^{31}P NMR

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A cDNA encoding the B isozyme of creatine kinase (CK_B) has been expressed in *Escherichia coli* from a fusion with *lacZ* carried by λgt11. Western blots indicate that a stable polypeptide with the appropriate mobility for the β-galactosidase-creatine kinase (β-gal-CK_B) fusion protein cross-reacts with both β-gal and CK_B antiserum. No significant CK activity is detected in control *E. coli;* however, extracts from cells containing the λgt11-CK_B construct have a CK activity of 1.54 ± 0.07 μmol/min per mg protein. The fusion protein appears to provide this activity because immunoprecipitation of protein with β-gal antiserum leads to a loss of CK activity from extracts. That the enzyme is active in vivo was demonstrated by detection of a phosphocreatine (PCr) peak in the ^{31}P NMR spectrum from *E. coli* grown on medium supplemented with creatine. As in mammalian brain and muscle, the PCr peak detected was sensitive to the energy status of the *E. coli*.

Galactosidase fusion, β-; Phosphocreatine; Energy metabolism

1. INTRODUCTION

Creatine kinase (CK) catalyses the reaction, phosphocreatine (PCr) + ADP = ATP + creatine (Cr) and is found at high levels in muscle, heart and brain [1]. The kinetics and enzyme mechanism of CK have been extensively studied for a number of years [3]. The reaction catalysed by CK is thought to enable PCr to act as a high energy buffer of ATP levels. Indeed, ^{31}P NMR studies of intact heart, muscle, and brain detect a drop in PCr prior to a fall in ATP when energy metabolism is compromised [4]. Activity is so high in muscle, heart, and brain that the reaction is believed to be in equilibrium. Thus this reaction can be used to estimate levels of free ADP in cytoplasm from measured levels of PCr, ATP, and Cr [2]. Localization of CK to myofibrils and mitochondria has led to the proposal that the CK reaction acts as a shuttle of high energy phosphates [5–7]; this proposal has not met with unanimous acceptance [8,9]. The ability to genetically alter the activity and levels of expression of CK would greatly aid studies of enzyme mechanism as well as its physiological role.

In addition to interest in the role of CK in energy metabolism, a number of workers are studying the tissue and developmental specificity of expression of the different isozymes of CK [10–12]. During development of muscle, for example, expression of the B isozyme is turned off and expression of the M isozyme is switched on. Expression of the M isozyme occurs concurrently with the expression of a number of other muscle specific genes. In order to study the regulation of CK genes a number of clones have been described [10–12]. These clones have been obtained primarily by antibody cross-reaction. Sequence information has verified that they encode CK. Here we report that the B isozyme

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of CK (CK_B) from mouse brain is active when expressed as a fusion protein with β-galactosidase (β-gal) in *Escherichia coli*. Furthermore, in *E. coli* containing CK activity a peak due to PCr can be detected in the ^{31}P NMR spectrum.

2. MATERIALS AND METHODS

λgt11 phage containing a full length cDNA coding for CK_B from mouse brain (λgt11-CK_B) was a kind gift from Dr P. Benfield (Dupont). The cDNA was cloned into the *Eco*RI site of λgt11 [12], thus expression is under control of the *lacZ* promoter and a fusion protein between β-gal and CK (β-gal-CK_B) should be expressed. λgt11 lysate was mixed with *E. coli* strain XK1502 and plated under conditions selective for formation of a lysogen [13]. XK1502 is a lac repressor$^-$ strain [14], thus expression of the fusion protein should be constitutive. Candidates were selected and tested for the presence of phage by cross streaking against λvir and λcI at 32 and 42°C.

E. coli were grown in Luria broth (LB) at 32°C. Typically 20 ml of an overnight culture were inoculated into 1 l of broth and grown with aeration to an absorbance of 1 at 600 nm. For NMR experiments the LB was supplemented with 40 mM creatine (Sigma). For gels, determination of enzyme activity and immunoprecipitation experiments, *E. coli* were spun at 1700 × *g* for 10–15 min and resuspended in 0.2 vol. of 10 mM Tris buffer, pH 7.4, containing 130 mM KCl, 10 mM NaCl, and 1 mM $MgCl_2$. This suspension was sonicated using a microtip at a setting of 6.5 for 30 s in a Branson sonicator. Sonicated material was stored on ice and used within 2 h for CK assays and immunoprecipitation or boiled for polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE was performed with a 7–16% linear acrylamide gradient and stained with Coomassie blue or proteins were transferred to nitrocellulose and detected with Western blotting. Polyclonal β-gal (Research Plus, Inc.) or polyclonal human CK_B (Calbiochem) rabbit antiserum was used as the primary antibody. Blots incubated with β-gal antiserum were then treated with biotinylated goat anti-rabbit IgG as a secondary antibody followed by an incubation with a solution of complexed aviden DH-biotinylated horseradish peroxidase (Vector Labs) [14]. Blots incubated with CK_B antiserum were washed with alkaline phosphatase conjugated goat anti-rabbit IgG (Promega Biotech). Visualization of proteins on Western blots in each case was done as appropriate for the enzyme involved in the detection system.

Immunoprecipitation was performed using protein A on active Staph (*Staphylococcus aureus*; Boehringer Mannheim). *E. coli* extract (25 μl) was pretreated with Staph (15 μl) and then incubated with varying amounts of β-gal antibody for 45 min. 15 μl of activated Staph was added and the solution was left on ice for 10–15 min. The resulting precipitate was spun down and the supernatant assayed for CK activity. CK assays were performed using Sigma assay kit no.520. The assay detects the formation of creatine from PCr + ADP catalysed by CK. Creatine is detected by reaction with naphthol + diacetyl to form a pink-red colored complex. The amount of color formed (absorbance at 520 nm) is proportional to CK activity. Protein concentration was determined using an assay based on Coomassie blue staining (Sigma).

NMR experiments were performed on a Bruker 4.7T/40 cm Biospec II spectrometer operating at a phosphorus frequency of 81 MHz. *E. coli* from 1 l of A_{600} = 1 cells were spun at 2600 × *g* for 15 min and resuspended in 15 ml of a solution containing 50 mM Tris (pH 7.4), 70 mM NaCl, 40 mM creatine, 1 mM inorganic phosphate, 1 mM $MgCl_2$, and 20 mM glucose. This suspension was placed in a homebuilt NMR probe which stirred the solution with an air driven mixer and oxygenated the cells by blowing 100% oxygen above the surface at a rate of 1 l/min. This technique has been shown to keep suspensions of cells well oxygenated [15]. Glucose additions were made every half hour to maintain nutrient supply to the *E. coli*.

3. RESULTS

Fig.1A shows the results of SDS-PAGE on *E. coli* control and γgt11-CK_B lysogen cell extracts. A band which runs at the appropriate molecular mass to be the β-gal-CK_B fusion (~160 kDa) is detected in cells containing λgt11-CK_B but not in controls. This band cross-reacts with both anti β-gal and anti CK_B antibodies (fig.1B). There is a large amount of background staining with the CK antibody; however, the band at the molecular mass of the fusion protein shows up only in *E. coli* containing λgt11-CK_B. No protein with the same gel mobility as CK cross-reacts with the CK_B antibodies.

Extracts from *E. coli* containing phage show an appreciable amount of CK activity (1.54 ± 0.07 μmol PCr hydrolysed per min per mg protein at 37°C) while control cells show no significant detectable CK activity (0.01 ± 0.02 μmol/min per mg protein). To show that the measured CK activity is associated with a β-gal fusion protein, immunoprecipitation with anti β-gal antibodies was performed. These results are summarized in table 1. Analysis of these results is complicated by the fact that the rabbit antiserum and the Staph contains a significant level of apparent CK activity. Values of CK activity are given for *E. coli* extract + Staph and Staph alone for different amounts of added anti β-gal. Upon subtraction of the background it can be seen that greater than 98% of the detected CK activity is lost upon precipitation. These results indicate that a fusion between β-gal and the amino terminal of CK retains CK activity.

That the protein is active in intact *E. coli* as well as in extracts is demonstrated by detection of PCr in *E. coli* grown in media containing creatine using ^{31}P NMR. The only known way to produce PCr is via the reaction catalysed by CK. Fig.2A shows the

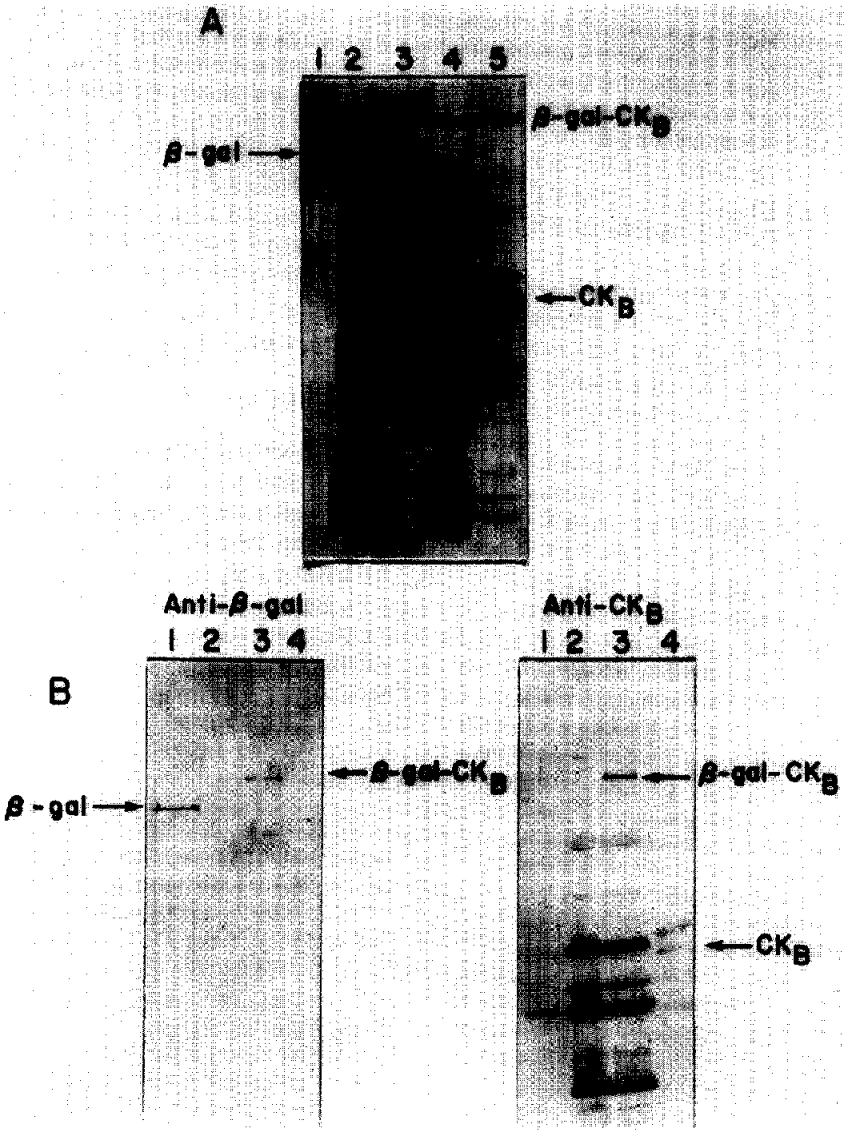

Fig.1. SDS-PAGE and Western blots of *E. coli* containing λgt11-CK_B and control. (A) Coomassie stained gel. Lanes are: 1, β-gal; 2, XK1502; 3, molecular mass markers; 4, XK1502 with λgt11-CK_B, the fusion protein is indicated by an arrow; 5, Sigma CK_B from rabbit brain. The band running at the proper molecular mass is labeled CK_B, the other bands are presumably impurities and degradation products. (B) Western blots stained with anti β-gal and anti CK_B. Lanes: 1, β-gal; 2, XK1502; 3, XK1502 with λgt11-CK_B; 4, Sigma rabbit brain CK_B. The anti CK_B serum cross-reacted with a number of proteins as well as with CK_B, even after prebinding in XK1502 extract. Both antibodies stain the fusion protein indicated with an arrow.

spectrum obtained from *E. coli* which did not contain λgt11-CK_B. The region between the peak due to phospho*enol*pyruvate (PEP) and the peak due to the gamma phosphate of nucleoside triphosphates (NTP) has been expanded in fig.2B. Fig.2C shows the same expanded region from *E. coli* which express β-gal-CK_B. A new peak is detected at 2.46 ± 0.02 ppm (n = 4) from the gamma NTP peak. This is where PCr resonates in rat brain and heart (2.5 ppm from gamma ATP, data not shown). The only peaks assigned to this region in ^{31}P NMR spectra from living cells have been from

Table 1

Effect of immunoprecipitation with anti β-galactosidase serum on creatine kinase activity

	μl of anti β-gal serum		
	0	0.2	10
λgt11-CK$_B$ + Staph	100[a]	15	130
Staph	12	15	129
Difference	88	0	1

[a] Activities are given as percents

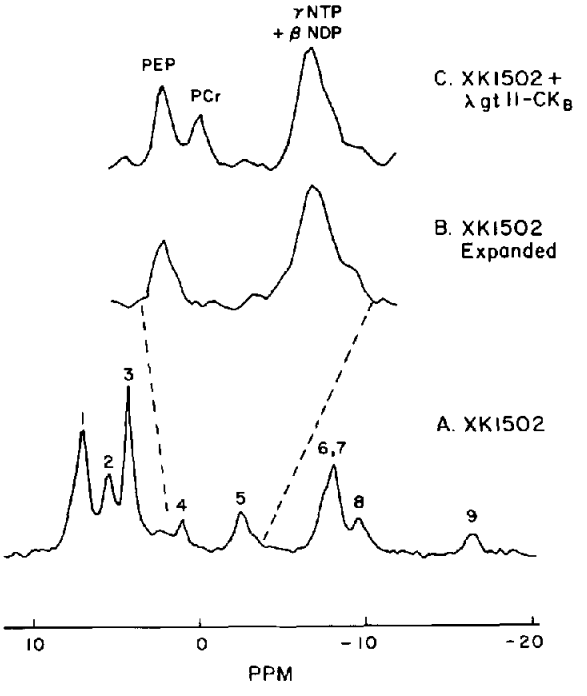

Fig.2. ^{31}P NMR spectra obtained from suspensions of *E. coli* grown on creatine. (A) Complete spectrum obtained from XK1502. (B) Same spectrum as in panel A, but with the region between phospho*enol*pyruvate (PEP) and the gamma phosphate of NTP expanded. (C) Expanded region from XK1502 with λgt11-CK$_B$. The peak due to PCr is labeled. Spectra were obtained with a 90° pulse, 2 s recycle time and 1024 transients were signal averaged. 10 Hz exponential linebroadening was applied prior to Fourier transformation. Peaks are: 1, phosphomonoesters: 2, intracellular inorganic phosphate; 3, extracellular inorganic phosphate; 4, phospho*enol*pyruvate; 5, the gamma phosphate from NTP + the beta phosphate from NDP; 6, the alpha phosphate from NTP and NDP; 7, NAD(P) + NAD(P)H; 8, UDP-sugars; 9, the beta phosphate from NTP.

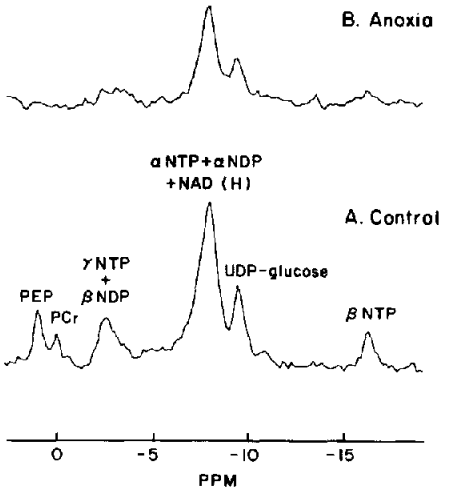

Fig.3. ^{31}P NMR spectra from *E. coli* containing CK$_B$ activity. (A) Spectra are from a well oxygenated suspension and (B) is from the same suspension 15 min after stopping the supply of oxygen. Spectra were acquired with a 90° pulse, 1 s recycle time, and 512 transients were signal averaged. 10 Hz exponential linebroadening was applied prior to Fourier transformation. Peaks are as labeled.

PCr or phosphoarginine [16]. That *E. coli* containing the fusion protein do produce PCr was verified by enzymatic analysis of perchloric acid extracts. Grown in 40 mM creatine, *E. coli* contain 0.49 ± 0.03 μmol of PCr per mg protein.

In tissues which express CK, the PCr level is sensitive to the energy status of the cell. Inhibition of ATP formation by anoxia leads to a rapid fall in PCr and ATP levels in these tissues. PCr produced in *E. coli* responds similarly to mammalian tissue, as shown in fig.3. The region from PEP to the beta phosphate of NTP has been plotted for well oxygenated *E. coli* and *E. coli* which had been deprived of oxygen for 15 min. Anoxia leads to complete loss of PCr and a large reduction in NTP levels. Therefore the response of PCr to anoxia in *E. coli* producing CK is similar to that found in mammalian cells.

4. DISCUSSION

Creatine kinase has been an extensively studied enzyme for a number of years and many details concerning enzyme mechanism and physiological role have been deduced. These studies would be facilitated if it were possible to manipulate CK ac-

tivity and cellular location in a number of different cell types. Here we show that the CK_B from mouse brain has activity when expressed as a fusion protein with β-gal in *E. coli*. Expression of CK_B leads to detectable levels of PCr and these levels respond to the energy status of the cell. Use of the equilibrium established by CK_B should allow determination of ADP levels in *E. coli* as has been done in mammalian muscle, heart and brain [2]. Levels measured in this way can be compared to those measured directly by NMR to test whether use of the CK equilibrium to estimate ADP is valid. *E. coli* is well suited for this experiment because ADP levels are high enough to be directly detected by NMR as opposed to mammalian tissue where ADP levels are too low to be directly detected [2,16].

The fact that CK_B retains activity in *E. coli* opens up possibilities for site directed or random mutagenesis studies of enzyme mechanism. In addition, because CK_B is active as an N-terminal fusion to β-gal there are a number of intriguing possibilities for directing the localization of CK. For example, CK could be localized to the cytoplasmic face of the inner membrane by making a fusion with a membrane protein of *E. coli*, such as the maltose transporter [17]. This would shed light on the role of membrane bound creatine kinase in energy metabolism. Membrane associated CK has been implicated as an important part of the CK shuttle [5–7]. Furthermore, CK_B could be localized to subcellular compartments, such as mitochondria, by fusion of the appropriate signal sequence to CK_B. Being able to manipulate CK activity in these ways should greatly enhance our understanding of the role of CK in energy transduction in a variety of cell types.

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