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A Comparative Study of Human Muscle and Brain Creatine Kinases Expressed in *Escherichia coli*

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We report the expression of the human muscle (CK-MM) and brain (CK-BB) creatine kinases in *Escherichia coli.* The proteins have been purified to apparent homogeneity and several of their physical and kinetic properties investigated. In the process, we have conclusively verified the correct DNA sequence of the genes encoding the respective isozymes, and determined the correct primary structure and mass of the gene products. Alignment of the primary sequences of these two enzymes shows 81% sequence identity with each other, and no obvious gross structural differences. However, Western blot analyses demonstrated the general lack of antigenic cross-reactivity between these isozymes. Preliminary kinetic analyses show the K_m and k_{cat} values for the creatine and MgATP substrates are similar to values reported for other isozymes from various tissues and organisms. The human muscle and brain CKs do not, however, exhibit the synergism of substrate binding that is observed, for example, in rabbit muscle creatine kinase.

KEY WORDS: Creatine kinase; human; expression; brain; muscle; purification; kinetics.

1. INTRODUCTION

Creatine kinase (CK; ATP:creatine *N*-phosphotransferase; EC 2.7.3.2)[4] catalyzes the reversible transfer of the γ-phosphoryl group of MgATP to creatine, resulting in the formation of phosphocreatine and MgADP. The enzyme plays a key role in cell energy metabolism (Watts, 1973) as well as in the transport of "high energy phosphates" (Jacobus, 1985). CK is a important clinical marker for a number of disease states including myocardial infarction (Wu, 1989) and muscular dystrophy (Gruemer and Prior, 1987).

The enzyme is most common in muscle and brain cells, although smaller amounts are found in other tissues. There are two cytosolic creatine kinase subunits, brain (B) and muscle (M), which associate to form the muscle (MM) and brain (BB) isozymes. In addition there is a heterodimeric isozyme (MB), and it is elevated levels of this form of the enzyme which are used as a marker for myocardial infarction. Finally, there are two forms of the enzyme associated with the inner membrane of mitochondria. These are referred to as the sarcomeric (Mi_s) and ubiquitous (Mi_u) isozymes and may exist either as dimers or octamers. Depending on the conditions, these forms are readily interconvertible (Mühlebach *et al.,* 1994), but, unlike the cytosolic CKs, no heteromers have been found *in vivo.*

Creatine kinase cDNAs, from species ranging from sea urchins to dogs, have been cloned and sequenced with considerable sequence homology being observed (Babbitt *et al.,* 1986; Mühlebach *et al.,* 1994). Human

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[4] Abbreviations: CK, creatine kinase; HMCK, human muscle creatine kinase; HBCK, human brain creatine kinase; CK-MM, creatine kinase muscle isozyme; CK-BB, creatine kinase brain isozyme; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; IEF, isoelectric focusing; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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muscle (Perryman *et al.,* 1986), human brain (Mariman *et al.,* 1989; Villareal-Levy *et al.,* 1987), and human mitochondrial (Haas and Strauss, 1990) cDNAs have all been cloned and sequenced. Predictably, all show a high degree of homology, with each encoding a protein of about 40 kDa. Recently, the human brain (HBCK) and human muscle (HMCK) isozymes have been expressed, at low levels, in CHO cells and some preliminary kinetic data obtained (Lin *et al.,* 1994). In order to obtain sufficient quantities of these human CK isozymes for X-ray studies, we have developed an system wherein both HMCK and HBCK are expressed at high levels in *E. coli.* This paper describes the expression and purification of these enzymes and compares their kinetic properties to the corresponding enzyme obtained from tissue preparations.

2. MATERIALS AND METHODS

2.1. Materials

Restriction and DNA modifying enzymes were purchased from Boehringer Mannheim and New England Biolabs. Reagents and enzymes for DNA amplification were purchased from Perkin-Elmer/Cetus and Stratagene. The plasmid vector, pET17b, was purchased from Novagen, Inc., while the bacterial strains, DH5α and BL21(DE3)pLysS, were obtained from BRLGibco and Novagen, respectively. Bacterial media was prepared using materials purchased from Difco. DNA sequencing of the human muscle and human brain CK cDNA was carried out by the dideoxy method (Sanger *et al.,* 1977) with the Sequenase kit (U.S. Biochemical), utilizing oligonucleotides synthesized by the Biomolecular Resource Center (University of California, San Francisco). Electrophoresis and protein assay reagents were from Bio-Rad and Amersham Pharmacia Biotech. All other reagents were of the highest quality available.

2.2. Subcloning of Human CK cDNA into the pET17/CK7 Vector

2.2.1. Human Muscle CK

A pET17b construct, containing rabbit muscle creatine kinase from pKTMCK (Chen *et al.,* 1991), was prepared and denoted pET17b/CK7 (Chen *et al.,* 1996). This construct contained a *Sal*1 site, downstream of the *Nde*1 site in pET17b.

The cDNA-derived HMCK gene, cloned into the mammalian expression vector pSG5 (Lin *et al.,* 1994), was obtained from Dr. M. B. Perryman (University of Colorado). The polymerase chain reaction (PCR) was used to engineer an *Nde*I restriction site (CATATG) at the initiating methionine and a *Sal*I site (GTCGAC) downstream of the 3′ terminus of the HMCK gene. The PCR was carried out on a Perkin Elmer/Cetus DNA Thermal Cycler 480 using 25 ng of dsDNA and 25 pmol of each oligonucleotide primer. Initially, the DNA was denatured at 94°C for 1 min, followed by a hybridization at 30°C for 1 min, and then extension at 72°C for 2 min. This was carried out for a total of five cycles, after which the hybridization temperature was elevated to 60°C to increase the stringency of amplification, and the amplification continued for 30 cycles. The amplified products were purified with glass beads from the USBioclean Kit (U.S. Biochemicals) as per the manufacturer's instruction. The PCR product containing the HMCK gene with the new *Nde*1 and *Sal*I sites was then digested with these enzymes, purified, and ligated into the pET17b/CK7 vector, which had been similarly digested. The resulting construct, containing the HMCK gene, was designated pETHMCK and is shown with pertinent restriction sites in Fig. 1A.

2.2.2. Human Brain CK

A plasmid containing the human brain CK cDNA (Villareal-Levy *et al.,* 1987) was also obtained from Dr. M. B. Perryman. In order to insert the cDNA into the pET17b/CK7 vector, *Nde*I and *Sal*I restriction sites were engineered into the 5′ and 3′ ends of the gene as described for the human muscle CK gene. Subsequent restriction digests and ligation steps were also carried out as for the muscle gene. The resulting construct, shown in Fig. 1B, was designated pETHBCK.

2.3. Expression of Human Muscle and Brain Creatine Kinase

E. coli cells, BL21 (DE3)pLysS, made competent either by calcium chloride treatment (Dagert and Erlich, 1979) or by the procedure of Hanahan (Hanahan, 1983), were transformed with the appropriate pET construct and plated on LB/ampicillin/choramphenicol at 37°C. A single colony was picked from this plate and grown overnight in liquid LB/Ap/Cm medium at 37°C. The cells were pelleted, resuspended in fresh media, and used to inoculate 1 L of LB/Ap/Cm. After the culture had reached a cell density of $A_{600} \approx 1.8$, the cells were cooled to 30°C, then induced with 0.5 mM IPTG. Cells were harvested 4 h postinduction.

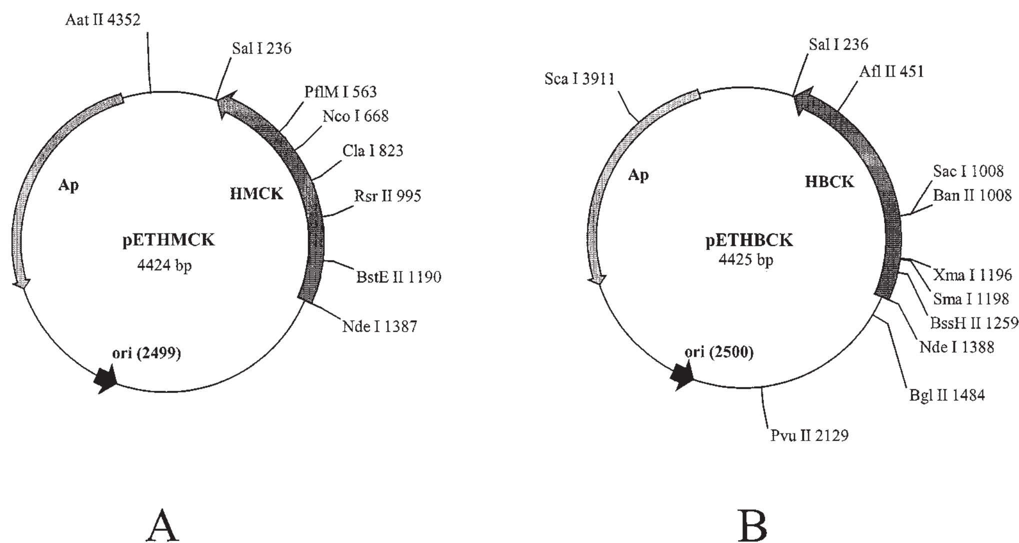

Fig. 1. Restriction maps of pETHMCK and pETHBCK used for the expression of human muscle and human brain creatine kinase, respectively. With the exception of the *Nde*1 and *Sal*1 sites, used in the cloning procedures, the restriction sites shown are unique to each vector.

2.4. CK Purification

2.4.1. Human Muscle CK

The purification scheme for the isolation of human muscle CK from *E. coli* was a modification of that described by Chen *et al.* (1991) for the purification of rabbit muscle creatine kinase. The cells were resuspended in buffer A (10 mM MES, 20 mM KCl, 0.1 mM PMSF, 1 mM DTT, pH 6.0). Cell lysis was achieved either by sonication (Ultrasonics Inc.) or by mixing with glass beads in a Beadbeater apparatus (BioSpec Products). In the previously published procedure, a 50%–75% ethanol fractionation step was included at this point. However, the ethanol fractionation was found to be too harsh a treatment for some of the CK isozymes, resulting in lower yields. The levels of CK expression were sufficiently high to allow us to bypass this step with no significant loss in activity or yield (data not shown). The lysed cells were centrifuged at 12,000 × *g* for 20 min, and the supernatant, containing the HMCK, was loaded directly onto a Blue-Sepharose 4B column (Amersham Pharmacia Biotech). After washing with 4–5 column volumes of buffer A, the enzyme was eluted with buffer B (10 mM TES, 1 mM DTT, 20 mM DTT, 20 mM KCl, 0.1 mM PMSF, pH 8.0). The eluted HMCK was concentrated and dialyzed against 50 mM TrisCl, pH 8.8, prior to application to MonoQ HR 10/10 column (Pharmacia Biotech). A linear NaCl gradient (0–100 mM, in the same buffer), was then introduced resulting in the elution of >99% pure HMCK at ~30 mM NaCl. The purified enzyme was stored at –20°C in 50 mM HEPES, 0.1 mM EDTA, 1 mM DTT, pH 7.0.

2.4.2. Human Brain CK

Cell lysis and initial chromatography over Blue Sepharose 4B was performed as described above for HMCK. The eluted sample was equilibrated in 50 mM TrisCl, pH 7.5, prior to chromatography over MonoQ HR 10/10 column. After washing in the same buffer, a 0–0.5M NaCl gradient was introduced, with the HBCK eluting at approximately 140 mM NaCl. Two peaks of equivalent specific activities were isolated, the latter containing >99% pure HBCK. Again, the purified enzyme was stored at –20°C in 50 mM HEPES, 0.1 mM EDTA, 1 mM DTT, pH 7.0.

2.5. SDS–PAGE, IEF–PAGE, and Western Blots

Protein samples were subjected to electrophoresis on a 12% SDS–polyacrylamide gel (Novex, San Diego, CA). Isoelectric focusing of CK isozymes was performed using a 5% nondenaturing polyacrylamide gel (pH 3–10, Novex) on a FBE-3000 Flat Bed Apparatus (Pharmacia) in accordance with manufacturer's instructions. The gels were prefocused for 1.5 h, the samples applied, and electrofocusing continued for an additional 2.5 h. The gel was fixed in a mixture of 5% sulfosalicylic acid

and 10% trichloroacetic acid before staining in 0.1% Coomassie blue R250.

Primary antibodies raised against the human muscle and brain CK in goat were purchased from Cortex Biochem., Inc., and alkaline phosphatase-conjugated secondary antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc. Other blotting reagents were purchased from Bio-Rad and used as per the manufacturer's instructions.

Western blotting was carried out using a Genie Electrophoretic Blotter (Idea Scientific Co.). Following 12.5% SDS–PAGE, samples of the purified CK isozymes (10 μg) were electrotransferred onto nitrocellulose. The nitrocellulose was then equilibrated in blocking buffer, 1% milk powder in 1× Tris-buffered saline +0.1% Tween 20 (1× TTBS) for 1 h at room temperature. After washing for 10 min with TTBS, one nitrocellulose strip containing both muscle and brain CKs was incubated for 1 h with a 1:500 dilution of goat α-human brain CK antiserum. A second strip was incubated with goat α-human muscle CK purified IgG at a concentration of 1:5000 in blocking buffer. The nitrocellulose strips were then washed with Tris-buffered saline +0.1% Tween 20, prior to incubation with the secondary rabbit α-goat FC conjugated to alkaline phosphatase. The blots were washed again for 3 × 5 min in TTBS, before development with the alkaline phosphatase conjugate substrate kit.

2.6. Mass Spectrometry

Human muscle and brain CKs, prepared as 20 μM solutions in methanol/water/acetic acid, were subjected to electrospray analysis on a modified Extrel Waters FTMS-2000 as described previously (Wood *et al.,* 1995*a*).

2.7. Enzyme Assays and Other Methods.

Absorbance measurements were carried out on a Hewlett-Packard 8452A diode-array spectrophotometer. Protein concentrations were determined using a modified Bradford assay (Bradford, 1976), with bovine serum albumin as the protein standard (Pierce). In addition, an extinction coefficient ($\varepsilon_{280(0.1\%)}$) of 0.88 (Cleland, 1979; Kuby *et al.,* 1962) was used to determine protein concentrations of the pure enzyme samples. Creatine kinase activity was determined either by the coupled assay system of Tanzer and Gilvarg (1959) or by a pH-stat (Radiometer Copenhagen) assay. Determination of the kinetic constants was carried out under the buffer conditions of Morrison and James (1965), and the data analyzed with the programs of Cleland (1979).

3. RESULTS AND DISCUSSION

3.1. DNA Sequence Verification

In the literature, two different DNA sequences for the human muscle creatine kinase gene have been reported (Perryman *et al.,* 1986; Trask *et al.,* 1988). In the latter report, several possible errors in the Perryman *et al.* (1986) gene sequence were alluded to. These discrepancies resulted in the following changes in the amino acid sequence: Ile_{47}Thr (whereby the Perryman sequence translated to an *isoleucine,* and the Trask sequence suggested *threonine*), P_{130}R, Q_{193}L, H_{210}D, P_{215}R, and AM_{324}G. Alignment of the primary sequence of several CK isozymes and other structural homologs (Mühlebach *et al.,* 1994) also suggest possible errors in the Perryman *et al.* (1986) sequence. For instance, threonine is a conserved residue in *all* muscle, brain, and mitochondrial CK sequences at position 47. Similarly, R_{130}, L_{193}, D_{210}, R_{215}, and G_{324}, are all highly conserved residues. While the conservation of these residues across several creatine kinase isozymes does not preclude the existence of other amino acid residues at those positions in HMCK, it does suggest that the Trask *et al.* (1988) sequence is more likely to be correct.

Two DNA sequences have also been reported for the human brain isozyme (Mariman *et al.,* 1989; Villareal-Levy *et al.,* 1987). Again, both sequences are at odds, with the discrepancies arising at several positions that show significant GC compressions. These base changes result in the translation of different amino acid residues in the human brain CK. Once again, an alignment of several CK and structural analogs was valuable for suggesting what may be the correct sequence. Such an analysis revealed that several of the residues are highly conserved and suggests that the original sequence (Villareal-Levy *et al.,* 1987) may be in error. These residues include Leu_{41} (which was previously translated as a valine), Gly_{97} (Arg), Gly_{98} (Arg), His_{105} (Asp), Arg_{131} (Ala), Arg_{214} (Ala), Gly_{215} (Arg), and His_{295} (Asp).

In light of these discrepancies, we have determined the DNA sequence of the human muscle CK gene, both in pGS5 (Lin *et al.,* 1994), as well as that in our *E. coli* expression construct pETHMCK. The results from both vectors were identical and are fully consistent with the

sequence of Trask *et al.* (1988). We also determined the DNA sequence of the cDNA-derived gene encoding the human brain CK isozyme from the original construct (Villareal-Levy *et al.,* 1987), as well as from our *E. coli* expression plasmid, pETHBCK. Again, the DNA sequences that we obtained from both of these constructs are identical and are consistent with the gene sequence derived from the genomic DNA by Mariman *et al.* (1989).

The correct translation of both human muscle and brain creatine kinase genes is shown in Fig. 2. The amino acid sequences demonstrate the high degree of homology that exists between the two isozymes (81%). However, the degree of homology is even more striking when the CK muscle isozymes are compared to one another, or when the brain isozymes are compared with one another (Babbitt *et al.,* 1986; Mühlebach *et al.,* 1994). Human and rabbit muscle creatine kinases, for example, are about 95% identical (Babbitt *et al.,* 1986).

The amino acid sequence of HMCK (Fig. 2) translates to a protein with a predicted subunit MW of 43,098 Da, whereas the sequence of Perryman *et al.* (1986) yields a predicted subunit MW of 42,911 Da. The observed mass of the cDNA-derived HMCK, as determined by Fourier transform mass spectrometry utilizing an electrospray ionization source (McLafferty, 1995; Wood *et al.,* 1995*a*), was shown to be 42,969 ± 2 Da. This is consistent with the HMCK sequence in Fig.2, lacking its N-terminal methionine residue (42,968 Da). Similarly, the predicted MW of HBCK from the Villareal-Levy *et al.* (1987) studies is 42,622 Da, whereas that derived from the sequence of Mariman *et al.* (1989) is 42,512 Da. The observed mass of the cloned, *E. coli*-expressed HBCK was shown to be 42,513 ± 2 Da. Thus, the observed masses of both the human muscle and the brain CK isozymes, overexpressed in a soluble form in *E. coli,* are consistent with the masses of the predicted proteins translated from the respective genes. In addition, these mass spectrometry results show that the PCR amplification and subsequent subcloning of the genes into an *E. coli* expression vector did not compromise the integrity of the resultant gene product.

Overall, the results from our independent DNA sequence determination of both CK genes and the mass spectral analyses on the purified proteins provide separate and distinct evidence for suggesting which DNA sequences correctly encode the brain CK (Mariman *et al.,* 1989) and the muscle CK isozymes (Trask *et al.,* 1988).

A

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1     MPFGNTHNKF KLNYKPEEEY PDLSKHNNHM AKVLTLELYK KLRDKETPSG
51    FTVDDVIQTG VDNPGHPFIM TVGCVAGDEE SYEVFKELFD PIISDRHGGY
101   KPTDKHKTDL NHENLKGGDD LDPNYVLSSR VRTGRSIKGY TLPPHCSRGE
151   RRAVEKLSVE ALNSLTGEFK GKYYPLKSMT EKEQQQLIDD HFLFDKPVSP
201   LLLASGMARD WPDARGIWHN DNKSFLVWVN EEDHLRVISM EKGGNMKEVF
251   RRFCVGLQKI EEIFKKAGHP FMWNQHLGYV LTCPSNLGTG LRGGVHVKLA
301   HLSKHPKFEE ILTRLRLQKR GTGGVDTAAV GSVFDVSNAD RLGSSEVEQV
351   QLVVDGVKLM VEMEKKLEKG QSIDDMIPAQ K*
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B

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1     MPFSNSHNAL KLRFPAEDEF PDLSAHNNHM AKVLTPELYA ELRAKSTPSG
51    FTLDDVIQTG VDNPGHPYIM TVGCVAGDEE SYEVFKDLFD PIIEDRHGGY
101   KPSDEHKTDL NPDNLQGGDD LDPNYVLSSR VRTGRSIRGF CLPPHCSRGE
151   RRAIEKLAVE ALSSLDGDLA GRYYALKSMT EAEQQQLIDD HFLFDKPVSP
201   LLLASGMARD WPDARGIWHN DNKTFLVWVN EEDHLRVISM QKGGNMKEVF
251   TRFCTGLTQI ETLFKSKDYE FMWNPHLGYI LTCPSNLGTG LRAGVHIKLP
301   NLGKHEKFSE VLKRLRLQKR GTGGVDTAAV GGVFDVSNAD RLGFSEVELV
351   QMVVDGVKLL IEMEQRLEQG QAIDDLMPAQ K*
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Fig. 2. Amino acid sequence of (**A**) human muscle and (**B**) human brain creatine kinase.

3.2. Purification and Characterization of Recombinant CK Isozymes

A simple, two-step purification scheme, which was essentially a modification of that used for rabbit muscle CK (Chen *et al.,* 1991), was developed for both isozymes. Following cell lysis and removal of insoluble cellular debris, the enzyme was purified by chromatography over Blue-Sepharose, followed by chromatography over a MonoQ resin. Figure 3 shows the SDS–PAGE analysis of a typical purification of HBCK and provides an indication of the purity of the final product The high yield of CK per liter of culture media allows us to bypass the ethanol precipitation step used in earlier creatine kinase purification schemes (Chen *et al.,* 1991; Kuby *et al.,* 1954; White *et al.,* 1992). With the current purification scheme, routine yields of 15–20 mg pure enzyme/L of culture were obtained for the HBCK isozyme, while even higher yields of 50–75 mg/L were obtained for the HMCK preparations.

Both rabbit muscle (Chen *et al.,* 1991; Maggio and Kenyon, 1977) and rabbit brain (Armstrong *et al.,* 1977) creatine kinase appear to be predisposed toward formation of sample microheterogeneity. Indeed, the heterogeneity of some isozymes has been used as a diagnostic marker in various disease states (Apple, 1989). It was

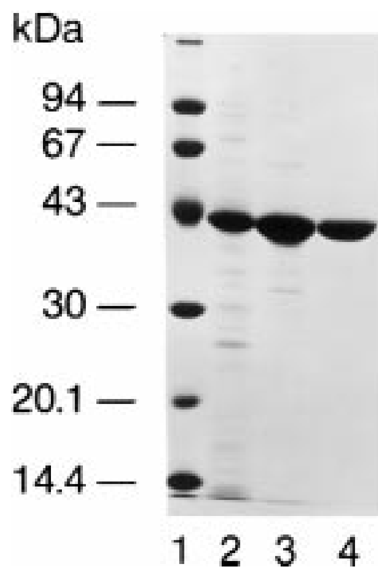

Fig. 3. SDS–PAGE analysis of recombinant human brain CK-BB protein samples after each purification step. Lane 1: Novex low-molecular-weight markers; lane 2: crude cell extract of induced BL21 (DE3)/pETHBCK; lane 3: CK-containing fraction after TES buffer elution of Blue Sepharose column; lane 4: after MonoQ column.

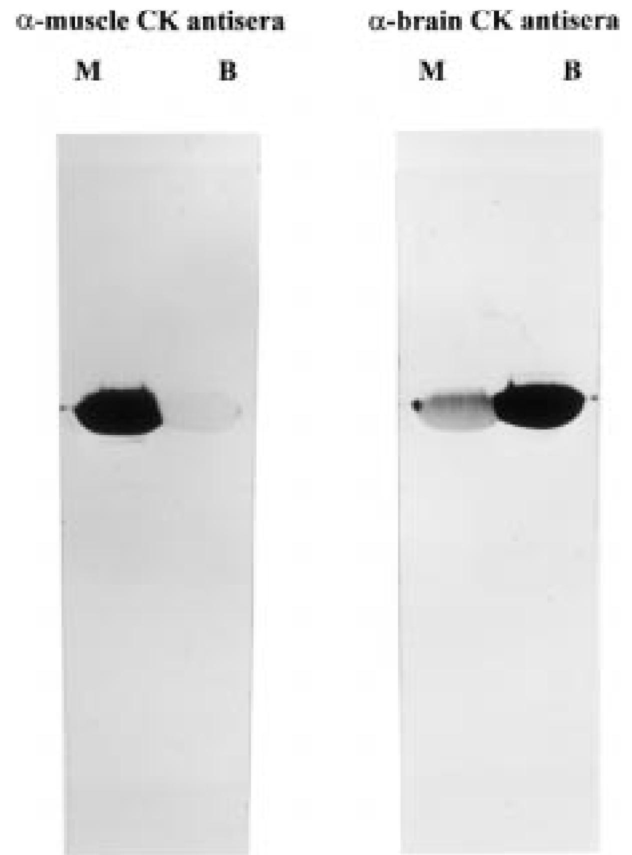

Fig. 4. Native IEF–PAGE (pH 3–10) analysis of recombinant human brain and human muscle CK isozymes. IEF standards (Novex) and CK isozymes (10 μg) were stained with Coomassie blue R250. Lane 1: IEF standards; lane 2: human brain CK-BB; lane 3: human muscle CK-MM.

suggested that the multiple forms may arise as artifacts of the purification (Chen *et al.,* 1991), and it was of interest to determine whether removal of the ethanol precipitation step would reduce the level of heterogeneity. Figure 4 shows a 5% isoelectric focusing gel of MonoQ-purified samples of the human brain and muscle isozymes. Both samples demonstrate significant heterogeneity. Using the sequence data shown in Fig. 2, the isoelectric point (pI) of human brain CK is predicted to be 5.37. However, the gel shows that there at least two major isoforms, with isoelectric points ranging from 4.9 to 5.4. Similarly, the predicted pI of human brain CK is 7.26. For the latter isozyme, the gel is even more striking, as a family of at least four isoforms with pI ranging from 6.9 to 7.8 can be seen. As in the case of the rabbit muscle CK, these isoforms were found regardless of the protein source (tissue-purified or expressed in *E. coli*), and all of the various isoforms were shown to have CK activity using a CK activity stain (White, 1996). Clearly the ethanol precipitation step, while it may have an influence on the overall yield of the purification, is not the sole source of the heterogeneity, and it is likely that deamidation is playing a significant role (Wood *et al.,* 1995b). This possibility is currently being investigated.

Despite their considerable sequence homology, there are significant differences between the brain and muscle isozymes. In addition to the IEF described above, experiments were also carried out to determine the level of antigenic cross-reactivity between the two isozymes. The results are shown in Fig. 5. In the first panel, primary antibodies to tissue-purified human *muscle* CK were reacted with purified, recombinant, human muscle and brain CK samples. The second panel shows the converse experiment, wherein antibodies raised against tissue-purified human *brain* CK are reacted with recombinant CK samples identical to those in the first panel. The most immediate result is that the antibodies raised against the tissue-purified enzyme recognize the *E. coli*-expressed samples, and therefore any possible posttranslational modifications of CK are not essential for antigenic recognition. Further, it demonstrates that the brain and muscle isozymes are immunologically distinct. This is consistent with previous work in our laboratory using salmon

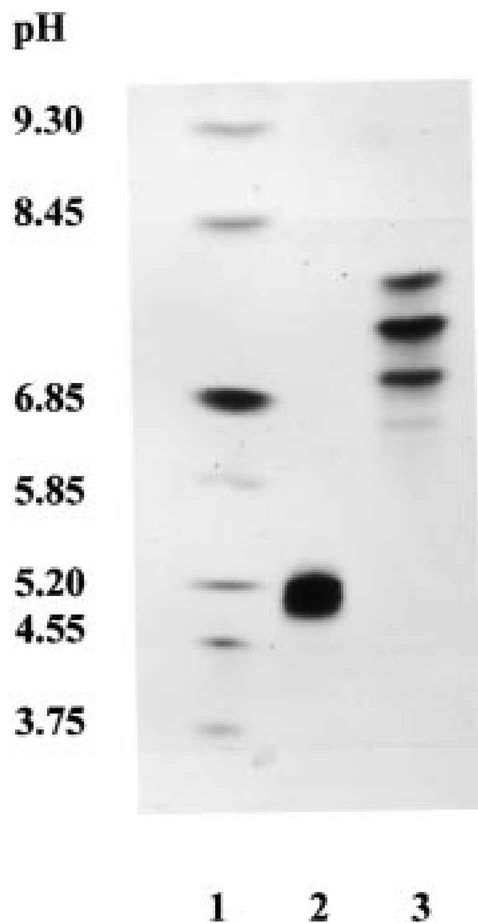

Fig. 5. Immunologic crossreactivity of recombinant human brain and muscle creatine kinase with antibodies raised against tissue-purified human CKs. Aliquots (10 μg) of purified recombinant human muscle (M) and human brain (B) creatine kinase were electrophoresed on 12.5% SDS–PAGE. After electroblotting with nitrocellulose the blot was cut in half. One half of the blotted protein was treated with α-brain CK antibodies and the other half was treated with α-muscle antibodies, as described in Materials and Methods.

brain and muscle CK (White *et al.,* 1992) as well as observations from other laboratories (Armstrong *et al.,* 1977; Geng *et al.,* 1989; Kato and Shimizu, 1986; Kato *et al.,* 1986). The figure also shows that, while the polyclonal antibodies raised to muscle CK are specific for the muscle isozyme, the antibodies raised against brain CK show some cross-reactivity with the muscle isozyme. Sensitive immunoassays using immobilized F(ab′)2 fragments of monospecific antibodies to both brain and muscle CK isozymes were found to have completely isozyme-specific recognition patterns (Kato and Shimizu, 1986, 1987; Kato *et al.,* 1986). The slight degree of cross-reactivity observed in our experiments may be due to a small amount of isozyme cross-contamination present in the tissue-purified brain CK used as the antigen for the production of the α-CK antibodies used in our Western blot analysis. Finally, polyclonal antibodies raised to recombinant CK-BB appeared to have identical cross-reactivity to recombinant brain CK as the antibodies raised against CK-BB purified from tissue (data not shown). This suggests that recombinant CK antigens may be acceptable for the purpose of generating α-CK antibodies for clinical or diagnostic use.

3.3. Kinetic Analysis

An initial kinetic analysis of the CK-MM and CK-BB isozymes was carried out for the reaction in the forward direction, i.e., formation of phosphocreatine and ADP. The data were obtained at pH 9.0, a pH value at which creatine kinase is expected to have a rapid equilibrium random bi–bi mechanism (Kenyon and Reed, 1983). The results are presented in Table I. The observed kinetic constants for both brain and muscle isozymes are similar to those reported for the rabbit muscle isozyme (Maggio and Kenyon, 1977), as well as for the human and brain isozymes expressed and isolated from COS cells (Lin *et al.,* 1994). Jacobs and Kuby (1980) carried out an analysis of autopsy tissue-purified CK-BB and CK-MM at pH 8.8 and, for comparison, their results are also shown in Table I. The V_{max} and K_m values are broadly in agreement, although the K_m value for the tissue-purified brain isozyme is marginally lower than that of its recombinant counterpart. It is not clear whether this difference is due to sample preparation or possibly to posttranslational modifications unique to the brain isozyme, such as phosphorylation (Chida *et al.,* 1990; Quest *et al.,* 1990; Wallimann *et al.,* 1992), which may have an effect on substrate binding.

It has been shown that the rabbit muscle CK exhibits synergism in the binding of the substrates, i.e., that the binding of the first substrate enhances the binding of the second substrate (Maggio and Kenyon, 1977; Morrison and James, 1965). It has been suggested that the synergism may be associated with substrate-induced conformational changes within the tertiary complex. The synergy can be identified by a decrease in K_m as compared to K_s for each substrate. The kinetic studies described here were carried out under conditions identical to those of Maggio and Kenyon (1977), who reported a threefold decrease in K_m vs. K_s for both creatine and MgATP. The results in Table I show that both K_m and K_s are similar for each of the recombinant human isozymes and indicate that neither of these

Table I. Comparison of Kinetic Constants for Recombinant and Tissue-Derived Creatine Kinases[a]

	Recombinant HBCK	Tissue HBCK[b]	Recombinant HMCK	Tissue HMCK[b]
K_aCr	6.0 ± 0.3	17 ± 2	14.6 ± 1.2	49 ± 14
K_mCr	4.9 ± 0.4	2.5 ± 0.1	9.5 ± 0.59	19 ± 2
K_aMgATP	0.99 ± 0.05	0.66 ± 0.04	1.2 ± 0.11	0.63 ± 0.05
K_mMgATP	0.81 ± 0.10	0.10 ± 0.01	0.89 ± 0.16	0.26 ± 0.08
k_{cat}(min^{-1})	12.9×10^3	7.15×10^3	9.21×10^3	8.67×10^3

[a] Concentrations are expressed in mM.
[b] From Jacobs and Kuby (1980).

isozymes appears to exhibit this synergism. Conversely, the K_m values of the tissue-purified CKs are significantly lower than the K_s values. Again it is not clear whether these differences are due to artifacts due to the purification process or possibly posttranslational modifications. Further studies aimed at full physical and kinetic characterization of the recombinant proteins are continuing in our laboratory.

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