Identification of a 43-kDa Polypeptide Associated with Acetylcholine Receptor-enriched Membranes as MM Creatine Kinase*

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Creatine kinase isoenzymes from *Torpedo californica* electric organ, skeletal muscle, and brain were purified and characterized. *Torpedo* electric organ and skeletal muscle creatine kinase have identical apparent Mr, electrophoretic mobility, and cyanogen bromide fragments. The electrophoretic mobility of the *Torpedo* creatine kinase was anodal as compared to mammalian MM creatine kinase. No creatine kinase isoenzyme with an electrophoretic mobility similar to mammalian BB creatine kinase was seen in any of the *Torpedo* tissues examined. Hybridization studies demonstrate the *Torpedo* electric organ creatine kinase to be composed of identical subunits and capable of producing an enzymatically active heterodimer when combined with canine BB creatine kinase. Creatine kinase from sucrose gradient-purified *Torpedo* electric organ acetylcholine receptor-rich membranes has an electrophoretic mobility identical with the cytoplasmic isoenzyme and an apparent Mr identical with mammalian MM creatine kinase. Western blot analysis showed *Torpedo* electric organ skeletal muscle creatine kinase and acetylcholine receptor-enriched membrane creatine kinase reacted with antiserum specific for canine MM creatine kinase. NH₂-terminal amino acid sequence determinations show considerable sequence homology between human MM, *Torpedo* electric organ, chicken MM, and porcine MM creatine kinase. The acetylcholine receptor-associated creatine kinase is, therefore, identical with the cytoplasmic form of the electric organ and is composed of M-subunits.

Acetylcholine receptor-rich membranes from *Torpedo* are enriched for three 43-kDa proteins referred to as the peripheral or ρ proteins as well as the five structural polypeptides of the acetylcholine receptor (for reviews see Refs. 1 and 2). One of the 43-kDa proteins has recently been identified as creatine kinase (ATP: creatine N-phosphotransferase, EC 2.7.3.2) (3, 4). This enzyme catalyzes the reversible transfer of a phosphate residue between ATP and creatine. The cytoplasm of mammalian and avian tissue contains two different types of enzyme subunits: muscle type (M) and brain type (B). Three dimeric isoenzymes are produced by various combinations of the two subunits (MM, MB, and BB). The identity of the creatine kinase isoenzyme associated with AcChoR-rich membranes from *Torpedo* remains controversial being identified as BB (3) and by others (4) as having electrophoretic mobility identical with human MM creatine kinase. Another similar creatine kinase from the electric organ of the electric eel (Electrophorus electricus) has been identified as the heterodimer MB (5). The physiological significance of the association of creatine kinase with the AcChoR is unknown. One of the three 43-kDa polypeptides has been identified as a protein kinase (6) but the physiological significance of creatine kinase association with AcChoR-rich membranes remains unclear. Resolution of the confusion surrounding the correct identification of the AcChoR-associated creatine kinase isofrom may help in elucidating the role of creatine kinase in AcChoR function.

Creatine kinase was purified from *Torpedo* electric organ, skeletal muscle, and brain for comparison to canine and human creatine kinase. Biochemical and immunological characterization of the creatine kinase from the electric organ, skeletal muscle, and AcChoR-rich membranes indicate that they are MM creatine kinases with a more anodal electrophoretic mobility than mammalian MM creatine kinase.

EXPERIMENTAL PROCEDURES

Materials

*Torpedo californica* electric organ, brain, and skeletal muscle were obtained from Pacific Biomarine (Venice, CA). Sephadex DEAE-A-50, Polybuffer Exchanger-94, Polybuffer-74, and SDS-polyacrylamide molecular weight standards were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). 125I was obtained from Amersham. Creatine kinase was assayed with Calbiochem Single Vial Reagent, Calbiochem-Behring Corp., and CK-NAC Reagent, Boehringer Mannheim Corp. Creatine kinase activity was assayed using the GEMENI miniature Centrifugal Analyzer, Electronucleonics, Inc. (Fairfield, NJ). Agarose gel electrophoresis reagents and apparatus were obtained from Corning Medical (Palo Alto, CA). Tris/Barbituric buffer was obtained from Gelman Sciences, Inc. (Ann Arbor, MI). Reagents for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories and Sigma. Rabbit muscle creatine kinase was obtained from Sigma. Cyanogen bromide was obtained from Pierce Chemical Co. (Rockford, IL). High performance liquid chromatography was performed on a Beckman 344 HPLC, Beckman (Palo Alto, CA), with a Bio-Sil TSK-250 column (300 x 7.5 mm) (Bio-Rad Laboratories). Molecular weight standards were obtained from United States Biochemical Corp. (Cleveland, OH). Protein A was purchased from Miles Laboratories (Elkhart, IN).

Methods

Purification of *Torpedo* Electric Organ and Skeletal Muscle Creatine Kinase—Electric organ and skeletal muscle creatine kinase was purified as previously reported (7) with the following modifications. Following ethanol precipitation, the preparation was dialyzed overnight against 50 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol. The preparation was centrifuged and concentrated to a volume of 30 ml in an Amicon chamber using a PM-30 membrane. The sample was applied to a DEAE-A-50 column (2.6 X 30 cm) which had been
Appropriate fractions were pooled and concentrated to 10 ml in an Amicon chamber previously equilibrated with 50 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptopethanol. The sample was applied to a chromatofocusing column (1 x 60 cm) packed with Polybuffer-94 equilibrated with 25 mM Tris/acetate, pH 8.3, 5 mM 2-mercaptoethanol. Elution was performed with a degassed solution of Polybuffer-74 HCl, pH 4.5. Two-ml fractions were collected and assayed for creatine kinase activity and appropriate fractions were pooled. The preparation was concentrated in an Amicon chamber using a PM-30 membrane. The concentrate was dialyzed overnight against three 4-liter changes of 25 mM Tris/acetate, pH 8.3, 5 mM 2-mercaptoethanol and reconcentrated. This step was repeated three times in order to remove excess salt and polybuffer. Specific activities were 675 units/mg and 320 units/mg of electric organ and skeletal muscle creatine kinase, respectively.

**Purification of Torpedo Brain Creatine Kinase**—Creatine kinase from the brain of *Torpedo californica* was purified as described by Ritter et al. (9). The specific activity was 80 units/mg. All other creatine kinases were purified as previously described (7).

**Creatine Kinase Assays**—Total creatine kinase activity was spectrophotometrically assayed at 30 °C according to the method of Rosalki (9) and expressed as international units per liter. Analysis was performed with and without creatine phosphate to exclude activity due to phosphocreatine kinases.

**Protein Determinations**—Protein concentrations were determined by the procedure of Lowry et al. (10).

**Polyacrylamide Gel Electrophoresis**—Polyacrylamide gel electrophoresis was performed according to the method of Laemmli (11).

**Isolation of AChoR-rich Membranes**—Acetylcholine receptor-rich membranes were purified and analyzed by the procedure of Burden et al. (12). The creatine kinase specific activity was 16-22 units/mg.

**Agarose Gel Electrophoresis**—Agarose gel electrophoresis was done using Corning agarose film and Tris/barbital buffer, pH 8.8 (Gelman). Following electrophoresis, the gels were overlayed with Rosalki reagent (Corning) or without (CK-NAC) creatine phosphate. The gels were photographed under UV illumination as previously described (13).

**Hybridization Experiments**—To determine whether *Torpedo* electric organ creatine kinase is a heterodimer or composed of identical subunits, the isoenzyme was denatured by exposure to 6 M guanidine HCl, followed by dialysis and subsequent transfer of the polypeptides to nitrocellulose was essentially that of Renart et al. (17). Following incubation with antibody to human creatine kinase and [3H]-labeled antibody, the filters were dried and exposed to Kodak X-Omat AR film with a DuPont Lightening-Plus intensifying screen at -80 °C for 12 h.

**Radioimmunoassays**—Radioimmunoassays were done as previously described (15).

**TSK Column Chromatography**—Molecular weight determinations using HPLC TSK column chromatography were done as described (16).

**Cyanogen Bromide Cleavage**—Cleavage of creatine kinase with cyanogen bromide was done as previously described (7).

**Protein Sequence Determination**—The amino-terminal amino acid sequence of *Torpedo* and human skeletal muscle creatine kinase was determined as described by Hewick et al. (18).

### RESULTS

Electrophoresis—Creatine kinase purified from *Torpedo californica* electric organ and skeletal muscle migrated as a single species with an apparent molecular weight of 43,000 on 10.8% SDS-polyacrylamide gel (Fig. 1). *Torpedo* electric organ, skeletal muscle, and brain creatine kinase were analyzed by agarose gel electrophoresis. The electrophoretic mobility of *Torpedo* skeletal muscle and electric organ creatine kinase are identical (skeletal muscle data not shown). Both migrate approximately equidistant between human MM and MB creatine kinase (Fig. 2). All of the mammalian MM creatine kinases examined remained much closer to the origin under these electrophoretic conditions. Creatine kinase purified from *Torpedo* brain was very unlike its mammalian counterpart (Fig. 3). There were two electrophoretically distinct species; one with a mobility identical with canine MM creatine kinase and another with a mobility identical with that of electric organ creatine kinase. The creatine kinase released from sucrose gradient-purified AChoR-rich membranes by incubation with electrophoresis buffer containing 0.1% Triton X-100 had an electrophoretic mobility identical with electric organ creatine kinase (Fig. 2, lane E). Incubation of human MM, BB, *Torpedo* electric organ, and brain creatine kinase with 0.1% Triton X-100 had no effect upon electrophoretic mobility (data not shown).

**Hybridization**—If *Torpedo* electric organ creatine kinase is a heterodimer, it would be expected to produce three isofoms upon denaturation in 6 M guanidine HCl, followed by dialysis to remove the denaturing agent and allow random rehybridization. When canine myocardial MM creatine kinase, BB creatine kinase, and *Torpedo* electric organ creatine kinase were denatured and allowed to rehybridize, only the original homodimers were reformed (Fig. 4) indicating that the *Torpedo* electric organ creatine kinase, like the canine MM and 14.4 kDa.
Enzymes. Creatine kinase isoenzymes were electrophoresed on 1% choline receptor-rich membranes by incubation with 0.1% Triton X-100, overlayered with Rosalki reagent, and photographed under UV illumination. The cathode is marked by - and the anode by +. The origin is marked by 0.

Fig. 2. Agarose gel electrophoresis of creatine kinase isoenzymes. Creatine kinase isoenzymes were electrophoresed on 1% agarose gels, overlayed with Rosalki reagent, and photographed under UV illumination. Lane A is canine myocardial MM creatine kinase, lane B is canine brain BB creatine kinase, lane C is Torpedo electric organ creatine kinase, lane D is Torpedo brain creatine kinase, lane E is creatine kinase released from sucrose gradient-purified acetylcholine receptor-rich membranes by incubation with 0.1% Triton X-100. The cathode is marked by - and the anode by +. The origin is marked by 0.

BB isoenzyme, consists of identical subunits. In contrast, when canine BB creatine kinase and Torpedo electric organ creatine kinase were mixed under identical conditions for denaturation and hybridization, both original homodimers are reformed as well as a species with an intermediate electrophoretic mobility. The putative heterodimer was similar to that of human MB creatine kinase as determined by this procedure was 66,000. This is in close agreement with the molecular weights obtained by this procedure for mammalian MM and mitochondrial creatine kinase (16).

Immunological Characterization—The assertion that Torpedo electric organ contains both MM and BB creatine kinase while only BB creatine kinase is associated with AChE membranes is primarily based upon immunological data obtained from Western blots of SDS-polyacrylamide gels (3). In attempts to verify these results, purified human skeletal muscle creatine kinase, brain BB creatine kinase, canine myocardial MM creatine kinase, brain BB creatine kinase, Torpedo electric organ, and skeletal muscle creatine kinase were electrophoresed on a 10.8% SDS-polyacrylamide gel. The peptides were transferred by diffusion to permit the simultaneous production of two nitrocellulose blots of the same gel. One of the replicas was reacted with antiserum to canine BB creatine kinase (Fig. 5A) and the other with antiserum to human MM creatine kinase (Fig. 5B). The antisera to MM creatine kinase reacted with human and canine MM creatine kinase as well as Torpedo electric organ and skeletal muscle creatine kinase. Even when the autoradiogram was overexposed, no reaction with human or canine BB creatine kinase was observed (Fig. 5B, lanes B and F). The same antisera has been previously shown to be specific for MM creatine kinase in a soluble phase radioimmunoassay (19). The antisera to BB creatine kinase reacted with all creatine kinases from the Torpedo electric organ and the skeletal muscle, as well as canine and human MM creatine kinases in the Western blot assay (Fig. 5A, lanes A and E). In contrast, the same BB creatine kinase antisera used for the Western blot analysis when used in a soluble phase radioimmunoassay was found to be specific for BB creatine kinase as demonstrated by the inability of the unlabeled MM creatine kinase to competitively displace 125I-labeled BB creatine kinase (Fig. 6). The lack of specificity of the BB antisera in the Western blot is probably due to MM and BB denaturation which gives rise to altered reactivity. The specific antisera to canine MM creatine kinase was used to probe another Western blot of purified Torpedo creatine kinases. Electrophoresis, transfer, and immunostaining were done as described above. The

Fig. 3. Agarose gel electrophoresis of creatine kinase isoenzymes. Creatine kinase isoenzymes were electrophoresed on 1% agarose gel, overlayed with Rosalki reagent, and photographed under UV illumination. Lane 1 is a mixture of human MM, MB, and BB creatine kinase; lane 2 is human MM creatine kinase; lane 3 is rabbit MM creatine kinase; lane 4 is mouse MM creatine kinase; lane 5 is canine MM creatine kinase; lane 6 is bovine MM creatine kinase; and lane 7 is electric organ creatine kinase. The cathode is marked by - and the anode by +. The origin is marked by 0.

Fig. 4. Hybridization analysis by agarose gel electrophoresis. Creatine kinase isoenzymes, singularly or in mixtures, were denatured in 6 M guanidine HCl, dialyzed, electrophoresed on 1% agarose gel, overlayed with Rosalki reagent, and photographed under UV illumination. Lane A is renatured canine MM creatine kinase, lane B is renatured electric organ creatine kinase, lane C is renatured canine BB creatine kinase, lane D is the renaturation of a mixture of electric organ and canine BB creatine kinase, and lane E is standard of human MM and MB creatine kinase. The cathode is marked by - and the anode by +. The origin is marked by 0.
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results illustrated in Fig. 5C indicate that the MM specific antiserum reacts with canine MM creatine kinase, Torpedo electric organ creatine kinase, and creatine kinase from AcChoR-rich membranes.

Amino Acid Sequence Homology—To further characterize the Torpedo electric organ creatine kinase, we compared the cyanogen bromide cleavage pattern of this isoenzyme to that obtained from canine myocardial MM creatine kinase and Torpedo skeletal muscle creatine kinase. The isoenzymes were cleaved with cyanogen bromide and electrophoresed on a 15% SDS-polyacrylamide gel. The results are shown in Fig. 7. The major polypeptide fragments produced from Torpedo electric organ and skeletal muscle creatine kinase are identical. The polypeptide fragment pattern produced from canine MM creatine kinase had polypeptide fragments which migrated identically with electric organ creatine kinase fragments as well as unique fragments. In order to assess the relationship of Torpedo electric organ creatine kinase to mammalian and avian MM creatine kinase, we determined the amino-terminal amino acid sequence of electric organ and human skeletal muscle MM creatine kinase by automated Edman degradation. The sequences obtained were compared to the protein sequence data for porcine MM creatine kinase (20) and the data for chicken MM creatine kinase as deduced from the cDNA nucleotide sequence (21). Human and porcine MM creatine kinase share 17 of 20 N-terminal residues. Human and chicken MM creatine kinase share 12 of 20 residues. Torpedo electric organ creatine kinase has 15 of 20 N-terminal residues which are identical with human, 15 of 20 identical with porcine, and 11 of 20 identical with chicken MM creatine kinase (Fig. 8).

DISCUSSION

Our data indicate that the creatine kinase of the cytoplasm of the Torpedo electric organ and skeletal muscle and that associated with AcChoR-enriched membranes are identical and are composed of identical subunits of the M-type. This was based on the observation that creatine kinase from the enriched AcChoR membrane and the electric organ and skeletal muscle cytoplasm reacted to antiserum specific for MM creatine kinase in a Western blot assay. Hybridization experiments showed that creatine kinase isoenzymes purified from both preparations upon dissociation followed by random association exhibited only the homodimer form. Additionally, creatine kinase from the electric organ in combination with canine or human BB creatine kinase did hybridize to form a heterodimer analogous to MB creatine kinase with interme-
These results would account for the previous identification of branes as BB creatine kinase. Thus, care should be taken in kinase antiserum was unaffected by antigen denaturation. BB creatine kinase antiserum, therefore, depends upon anti-tem (Fig. 6) did not show such specificity in a Western blot in a soluble competitive displacement radioimmunoassay system. The reason for this discrepancy between the molecular weight obtained by this procedure and the molecular weight predicted from SDS-polyacrylamide gel electrophoresis is 43,000. The absence of a higher molecular weight species is further evidence against the presence of a B-subunit. The molecular weight of electric organ creatine kinase as determined by TSK-chromatography was 67,000. This is in close agreement with molecular weights determined by the procedure for canine MM and mitochondrial creatine kinase (16). The reason for this discrepancy between the molecular weight obtained by this procedure and the molecular weight predicted from SDS-polyacrylamide gel (86,000) has not been examined by hydrodynamic techniques. However, the results are consistent with molecular asymmetry (21, 22).

The primary biochemical characteristic for the classification of mammalian and avian creatine kinase isoforms is electrophoretic mobility. Under electrophoretic conditions routinely used, BB creatine kinase is electronegative and migrates toward the anode. MM creatine kinase is less electronegative and remains close to the origin. MB creatine kinase migrates to a position intermediate between the other two forms (Fig. 3, lane A). Torpedo electric organ, skeletal muscle, and sucrose gradient-purified acetylcholine receptor-rich membrane creatine kinase all migrate as a single electrophoretic species with a mobility that was intermediate between that of human MM and MB creatine kinase. Thus, on the basis of the electrophoretic mobility without careful concomitant comparison to electrophoretic pattern of mammalian forms, the creatine kinase from the preparations may be misinterpreted as MB creatine kinase. None of the Torpedo tissue, including brain, in this study contained creatine kinase analogous to mammalian BB creatine kinase. The creatine kinase from the Torpedo brain exhibited two isoforms, one of which had electrophoretic mobility identical with canine and human MM creatine kinase and another minor species with a mobility identical with electric organ and skeletal muscle creatine kinase.

The previous identification of AcChoR-rich membrane creatine kinase as BB creatine kinase depended on immunological studies using Western blot analysis (3). Data presented in this study demonstrate how results based on the Western blot analysis may be misleading. Antiserum to BB creatine kinase which was shown to be specific for BB creatine kinase in a soluble competitive displacement radioimmunoassay system (Fig. 6) did not show such specificity in a Western blot assay. In addition to BB creatine kinase, the antiserum also reacted with canine and human MM creatine kinase as well as the creatine kinase isoenzyme from Torpedo electric organ and skeletal muscle (Fig. 5A). Antiserum to MM creatine kinase did not react with canine or human BB creatine kinase in the same Western blot assay (Fig. 5B). The specificity of BB creatine kinase antiserum, therefore, depends upon antigen conformation while the specificity of the MM creatine kinase antiserum was unaffected by antigen denaturation. These results would account for the previous identification of the creatine kinase associated with AcChoR-enriched membranes as BB creatine kinase. Thus, care should be taken in identifying creatine kinase isoenzymes as BB creatine kinase based upon Western blot analysis.

We have recently shown that mammalian M creatine kinase subunits have molecular weights of 43,000 and that B creatine kinase subunits have higher molecular weights which are unique for each species examined (6). The apparent molecular weight for Torpedo electric organ and skeletal muscle creatine kinase subunits as determined by SDS-polyacrylamide gel electrophoresis is 43,000. The absence of a higher molecular weight species is further evidence against the presence of a B-subunit. The molecular weight of electric organ creatine kinase as determined by TSK-chromatography was 67,000. This is in close agreement with molecular weights determined by the procedure for canine MM and mitochondrial creatine kinase (16). The reason for this discrepancy between the molecular weight obtained by this procedure and the molecular weight predicted from SDS-polyacrylamide gel (86,000) has not been examined by hydrodynamic techniques. However, the results are consistent with molecular asymmetry (21, 22).

The N-terminal amino acid sequence reported here for Torpedo electric organ creatine kinase (Fig. 8) shows considerable homology with that of human, porcine, and chicken M creatine kinase. Two recent publications (24, 25) have reported sequence data deduced from cDNAs for M creatine kinase from the electric organs of T. californica and T. maro†. The deduced N-terminal amino acid sequences are identical for the two cDNAs and they agree, with the exception of one residue, with the sequence data for T. californica reported here.

We conclude that the creatine kinase from Torpedo AcChoR-rich membranes is identical with the creatine kinase from skeletal muscle and electric organ. The isoenzyme is composed of identical subunits with an apparent molecular weight of 43,000 and a electrophoretic mobility intermediate to human MM and MB creatine kinase. Biochemical and immunological characterization show the subunits to be that of the M-type. The creatine kinase from the Torpedo electric organ cytoplasm has N-terminal amino acid sequence homology with other MM creatine kinases and should therefore be classified as MM creatine kinase. These data also pointed out the danger of classifying creatine kinase from lower vertebrates based solely upon electrophoretic mobility as compared to the isoenzymes from mammalian and avian species.

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REFERENCES

CHICKEN M Pro-Phe-Ser-Ser-Thr-His-Asn-Lys-His-Lys-Leu-Lys-Phe-Ser-Ala-Glu-Glu-Glu Pro-His-Leu-Ser-Lys
PORKINE M Pro-Phe-Gly-Ser-Thr-His-Asn-Lys-Tyr-Lys-Leu-Asn-Phe-Ala-Pro-Thr-Leu-Ser-Lys
HUMAN M Pro-Phe-Gly-Asn-Thr-His-Asn-Lys-Phe-Lys-Leu-Asn-Tyr-Lys-Pro-Glu-Glu-Glu-Pro-Leu-Ser-Lys
TORPEDO M Pro-Phe-Gly-Thr-His-Asn-Lys-Tyr-Lys-Leu-Asn-Ser-Ala-AAs-Leu-Glu-Glu-Pro

Fig. 8. A comparison of NH2-terminal sequence of MM creatine kinase from chicken, pig, human, and Torpedo electric organ. The chicken sequence is from Kwiatkowski et al. (21) and the pig sequence is from Takasawa et al. (20).
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