Creatine Kinase Isoenzymes in *Torpedo californica*: Absence of the Major Brain Isoenzyme from Nicotinic Acetylcholine Receptor Membranes†

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ABSTRACT: Creatine kinase, actin, and ν_1 are three proteins of M_r 43 000 associated with membranes from electric organ highly enriched in nicotinic acetylcholine receptor. High levels of creatine kinase are required to maintain adequate ATP levels, while actin may play a role in maintaining the synaptic cytoskeleton. Previous investigations have prompted the conclusion that postsynaptic specializations at the receptor-enriched membrane domains in electroplax contain the brain form of creatine kinase rather than the form of creatine kinase predominantly found in muscle. We have examined this conclusion by purifying *Torpedo* brain creatine kinase to virtual homogeneity in order to examine its immunochemical, molecular, and electrophoretic properties. On the basis of immunological cross-reactivity and isozyme analysis, the receptor-associated creatine kinase is identified to be of the muscle type. When the molecular characteristics of *Torpedo* brain and muscle creatine kinase are compared, the brain enzyme is positioned at a more basic pH during chromatofocusing and on two-dimensional gel electrophoresis (pI = 7.5–7.9). Furthermore, electrophoretic mobilities of the brain and muscle forms of creatine kinase differ in sodium dodecyl sulfate electrophoresis: the brain isozyme of creatine kinase has lower apparent molecular weight (M_r 41 000) when compared with the muscle enzyme (M_r 43 000). On the basis of the results of our current investigations, the hypothesis that the brain isozyme of creatine kinase is a component of the postsynaptic specializations of the *Torpedo californica* electroplax must be abandoned. Recent sequence data have established close homology between *Torpedo* and mammalian muscle creatine kinases. On the basis of electrophoretic criteria, our results indicate that a lower degree of homology exists between the brain isozymes.

Creatine kinase (CK)[1] has been identified as one of the components of the 43 000-dalton proteins copurifying with nicotinic AChR when purified in its membrane environment (Gysin et al., 1983; Barrantes et al., 1983a,b). An SDS electrophoresis band of 43 000 daltons, the ν-band (Hamilton et al., 1979), is the major nonreceptor subunit present in purified membranes that are otherwise highly enriched in the four integral subunits of the AChR. The 43 000-dalton proteins, which are not involved in any of the known receptor functions (Neubig et al., 1979; Moore et al., 1979; Elliott et al., 1980), can be removed from the membranes by extraction in alkali and are, therefore, peripheral rather than integral membrane proteins. The heterogeneous nature of the 43 000-dalton proteins was established on two-dimensional gels, which resolve more than seven spots in this molecular weight region (Saitoh & Changeux, 1980; Gysin et al., 1981). The multiple two-dimensional electrophoretic spots may be grouped into three distinct proteins, ν_1, ν_2, and ν_3, which were distinguished on the basis of their unique peptide maps and isoelectric focusing positions (Gysin et al., 1981). The major 43 000-dalton protein, ν_1, is almost exclusively membrane associated whereas CK (or ν_2) and actin (ν_3) are also major components of the cytosol. It has been established that ν_1 is localized on the cytoplasmic side of the postsynaptic specializations and is largely coextensive with the AChR (Froehner et al., 1981; Porter & Froehner, 1983; Nghiem et al., 1983; Sealock et al., 1984). Removal of 43 000-dalton proteins greatly increases the mobility of the receptor (Rousselet et al., 1979, 1982; Barrantes et al., 1980; Lo et al., 1980; Cartaud et al., 1981). These observations suggest that ν_1, which has recently been shown to bind actin on blots (Walker et al., 1984), may be involved in anchoring the receptor in the postsynaptic membrane and with elements of the cytoskeleton. No physiological role for CK at the synapse has been identified so far. The biochemical identification of ν_2 as CK was established by purifying the enzyme activity from the electroplax cytosol, providing a reliable amino acid composition (Gysin et al., 1983). The availability of the major electroplax CK amino acid composition assisted the verification of creatine kinase sequence data derived from full-length cDNA clones (Giraudat et al., 1984).

CK is a ubiquitous enzyme with a wide tissue distribution in vertebrates (Watts, 1973). It is found in especially high concentrations in muscle and brain where it plays a major role in homeostasis of the energy equilibrium by maintaining relatively constant levels of ATP during high-energy demand. In most of the species examined, CK is a dimeric enzyme with subunit molecular weights between 40 000 and 45 000. Two different isozymes, largely specific for skeletal muscle (MM) and brain (BB), are the most abundant forms of the enzyme (Eppenberger et al., 1967). A third form with the mixed heterodimer (MB) is found in heart and other smooth muscle tissue. A fourth isozyme with unique subunit composition has been isolated from mitochondria (Roberts & Grace, 1980). The bulk of the CK activity is found in the cytosol upon cell fractionation, and in the past most investigators have treated CK as a purely soluble enzyme. A fraction of CK, however,

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[1] Abbreviations: CK, creatine kinase (EC 2.7.3.2); M-CK, subunit of muscle-specific MM-CK isozyme; B-CK, subunit of brain-specific BB-CK isozyme; nAChR, nicotinic acetylcholine receptor; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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has been found to be associated with various intracellular compartments [for review, see Neumeier (1981)], such as the M line of myofibrils (Turner et al., 1973; Wallimann et al., 1977), the mitotic spindle apparatus of nuclei (Cande, 1983), and the cytoskeleton (Koons et al., 1982). In these contractile elements, CK may locally mediate the level of ATP required during the contraction process. The identification of CK in synaptosomal membrane fractions (Friedhoff & Lerner, 1977; Lim et al., 1983) suggested that the enzyme may also play a role at synapses in the central nervous system. However, no synaptic function has so far been shown to be mediated by CK.

Considerable uncertainty exists as to which isozyme is associated with AChR membranes. Earlier isozyme electrophoresis had identified ν_2 as the M form, and the membrane and cytoslic form of the enzyme were identical in peptide maps and migration on two-dimensional gels (Gysin et al., 1981, 1983). Sequencing data have also established that the *Torpedo* electroplax CK (West et al., 1984; Giraudat et al., 1984) shares close homology with the M form of chicken (Ordahl et al., 1984), rabbit (Putney et al., 1984), and rat (Benfield et al., 1984). A different situation is suggested from experiments using isozyme-specific antibodies that have been obtained against chicken M-CK and B-CK (Barrantes et al., 1983a,b; Wallimann et al., 1985). These authors found that the AChR membranes reacted with B-CK antibody, whereas the electric organ contained the isoform cross-reacting with M-CK antibody mainly in the cytosol. The enzymes from both compartments were purified and characterized, and the membrane-associated B-CK-like form was identified as ν_2 (Barrantes et al., 1985).

In this paper, we present data that further establish the AChR membrane associated CK to be the M isoform. Using an antiserum that has been prepared against purified electroplax CK, we show that strong immunological cross-reactivity exists with muscle fractions, but not with brain. This conclusion is further substantiated through isozyme analysis on electrophoresis plates. In addition, we have purified the major CK from *Torpedo* brain and show that its molecular properties differ significantly from the electroplax isozyme. The B-CK enzyme was found to be absent in AChR membranes. Portions of this work have been previously presented in abstract form (Gysin & Flanagan, 1984, 1985).

Experimental Procedures

Brain, Muscle, and Electroplax Preparations. Torpedo californica were obtained live from Marinus Inc., Westchester, CA. AChR-enriched membranes, alkaline extracts, and cytosol from the electric organ (electroplax) were prepared as previously described (Gysin et al., 1981, 1983). The whole brain was removed, frozen in liquid nitrogen, and subsequently stored at −56 °C until use. Dorsal muscle tissue was dissected from the tail section and treated the same. For fractionation, the brain or muscle tissue was homogenized with a Polytron mixer (Brinkmann Instruments) after addition of 1 mL/g wet weight of tissue of homogenization buffer, which consisted of 150 mM NaCl, 10 mM sodium phosphate, pH 7.4, 2 mM EDTA, and 2 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged for 10 min at 3000 rpm in a Sorvall SS-34 rotor; the pellet was rehomogenized in 0.5 volume of buffer and centrifuged as before. The pellet (low-speed pellet) was retained; the supernatants were combined and filtered through eight layers of cheesecloth and then centrifuged for 45 min at 40 000 rpm in a Ti-60 rotor. The high-speed pellet was retained, and the supernatant was recentrifuged for 30 min at 40 000 rpm and used as the cytosol fraction for isoenzyme experiments or chromatofocusing.

Isozyme Electrophoresis. Samples were diluted in 50 mM dithiothreitol and 0.1% bovine serum albumin, and 1–5 μL was pipetted into the wells of thin-layer agarose plates obtained from Corning, Palo Alto, CA (Electro-Trace special purpose electrophoresis film agarose, catalog no. 470024). The plates were placed in a flatbed electrophoresis unit (Bio-Rad) and connected with paper wicks to the buffer tanks containing 0.05 M MOPSO [3-(*N*-morpholino)-2-hydroxypropanesulfonic acid] buffer, pH 7.8. Electrophoresis was performed for 45 min at 400 V under cooling to 18 °C. The enzyme activity was visualized with the substrate kit from Corning (Cardiotrac-CK, catalog no. 470069) according to the manufacturer's instructions and photographed under UV illumination.

Chromatofocusing. A column of 1.5-cm diameter containing a 22-mL bed volume of Polybuffer Exchanger (PBE, Pharmacia) was equilibrated with chromatofocusing starting buffer, consisting of 25 mM Tris-HCl, pH 8.5. Brain cytosol was made 25 mM with 2-mercaptoethanol and dialyzed against starting buffer supplemented with 5 mM 2-mercaptoethanol; the precipitate that formed during dialysis was removed by centrifugation for 40 min at 45 000 rpm in a Ti-60 rotor. The sample was loaded on the column and eluted at 11 mL/h with 230 mL of 7% PB-96 (Polybuffer, Pharmacia), pH 6.5, followed by 260 mL of 12% PB-74, pH 5.0. Protein not eluted in the pH gradient was removed with 1 M NaCl, and the column was briefly rinsed with 8 M urea in 0.1 N HCl before reequilibration.

Affi-Gel Blue Chromatography. A column of 0.7-cm diameter, containing 2 mL of Affi-Gel Blue (Bio-Rad), was washed with 1.5 M NaCl and then equilibrated with 25 mM Tris-HCl buffer, pH 8.5, containing 2×10^{-6} M 2-mercaptoethanol, 2×10^{-4} M EDTA, and 5×10^{-3} M $MgCl_2$. The concentrated CK fraction from chromatofocusing was adsorbed on the column and eluted with a 0–1 M NaCl gradient in the same buffer. Fractions containing CK activity, eluting at 0.5 M NaCl, were pooled and concentrated on an Amicon YM-10 membrane.

Other Procedures. Determination of CK enzyme activity, two-dimensional and SDS gel electrophoreses, and immunoblotting were performed as described previously (Gysin et al., 1981, 1983). Protein determinations were made by the Coomassie Blue dye binding assay from Bio-Rad Laboratories with bovine serum albumin as a standard.

Results

CK from Electroplax Cross-Reacts Immunologically with Muscle but Not with Brain Fractions. We previously demonstrated that the soluble CK and the form bound to nAChR membrane are identical on the basis of their indistinguishable peptide maps (Gysin et al., 1981). Subsequently, we purified CK from the electroplax cytosol by using the newly introduced chromatofocusing procedure (Gysin et al., 1983). Although our original chromatofocusing conditions gave CK of high purity, some minor bands of higher molecular weight were still present, and estimates from densitometric scanning of SDS gels indicated a purity of 85% (Gysin et al., 1983). Upon modification of our original procedure by reducing the elution flow rate, we obtained electrophoretically homogeneous CK with a single chromatofocusing step. The highly purified CK preparation was injected into a rabbit, and a highly reactive antiserum was prepared. The antiserum was found to be monospecific for CK on immunoblots and has the capacity to precipitate radioiodinated CK under stringent conditions, viz., in the presence of detergents that minimize nonspecific adsorption (Gysin et al., 1983). Under these conditions 90% of

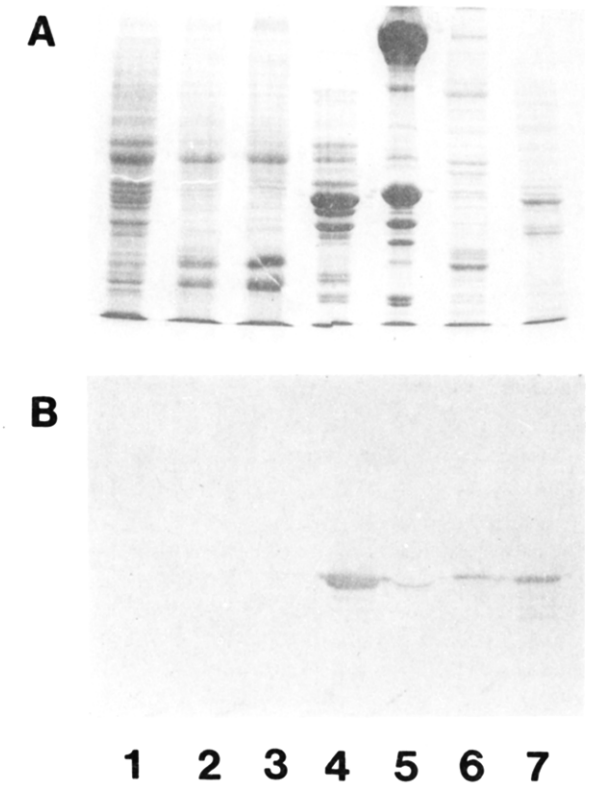

FIGURE 1: Reaction of antiserum against the major electroplax CK with fractions from brain and muscle. In order to assess the cross-reactivity of CK antiserum that had been raised against electroplax enzyme ν_2, we prepared soluble and particulate fractions from *Torpedo* brain andd muscle. SDS electrophoresis was then performed on 10% polyacrylamide gels, followed by immunoblotting on nitrocellulose filters. The top panel (A) is the Coomassie staining profile; the bottom panel (B) is an autoradiograph (5-h exposure) of an identical gel after being blotted onto nitrocellulose and subsequent incubation with CK antiserum (1:50 dilution) and visualization of the bound antigen with ^{125}I-labeled protein A. The lanes were loaded with the following: brain cytosol (1); the particulate fractions from brain, high-speed pellet (2) and low-speed pellet (3); muscle cytosol (4); muscle high-speed pellet (5); muscle low-speed pellet (6); electroplax cytosol (7). The samples from brain and muscle contained 40 μg of protein/lane; the sample from electroplax contained 20 μg.

^{125}I-CK, iodinated with the chloramine T method, is precipitated at dilutions of 1:50 and 1:100, and even at 1:20 000 serum dilution, the amount of ^{125}I-CK precipitated is still 10-fold above background (data not shown).

In order to determine whether the electroplax enzyme shares antigenic determinants with muscle or brain CK, we have investigated the reaction of our anti-electroplax CK antiserum with fractions from *Torpedo* brain and skeletal muscle (Figure 1). Strong reaction was observed with the muscle cytoplasm on immunoblots from SDS gels. Crude particulate fractions from muscle were also reactive, albeit at lower intensity. The antiserum strongly reacted with a band of M_r 43 000 in muscle that comigrates with electroplax CK. Also, several minor bands between M_r 30 000 and M_r 40 000, possibly due to proteolysis, were identical in the cytosol of muscle and electroplax. The anti-ν_2 CK antiserum did not react with the subcellular fractions derived from whole *Torpedo* brain.

Electroplax Membranes Contain Only the Muscle Enzyme. While the previous experiments clearly show the homology between the electroplax and the muscle enzymes, definitive evidence to rule out the possibility that the brain isoform was present in electroplax membranes was still needed. We therefore analyzed the isoenzyme patterns from various electroplax fractions together with brain and muscle by agarose

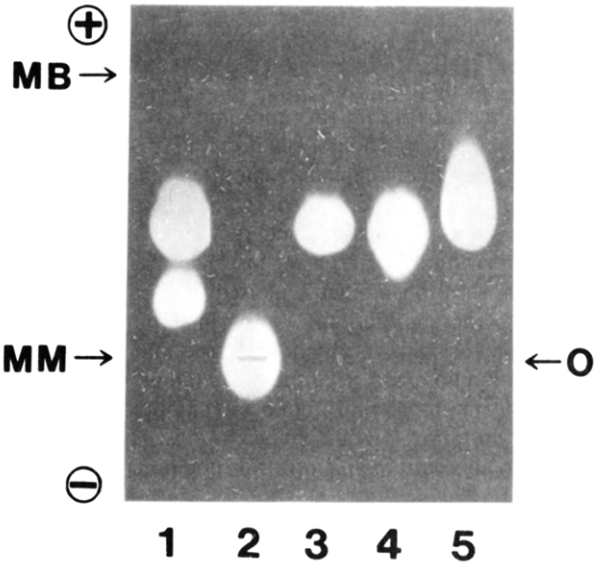

FIGURE 2: Identification of CK isozymes in *Torpedo* brain, muscle, and electroplax fractions. The samples were appropriately diluted and applied to a Corning agarose special purpose film. After electrophoresis for 45 min at 400 V, CK was visualized by means of the fluorescent reaction product produced by a coupled enzyme reaction procedure recommended by Corning. The arrow to the right marked (O) designates the origin of sample application; migration was from bottom (−) to top (+). The first three lanes show the cytosol fractions from electroplax (1), brain (2), and muscle (3). Lanes 4 and 5 show the enzyme from electroplax membranes: sucrose gradient purified AchR membranes were loaded in lane 4, while lane 5 contained an alkaline extract from affinity purified receptor containing membranes. For comparison, the position of the human isozymes is marked with arrows on the left side; the human muscle (MM) isozyme remains at the origin, and the human hybrid (MB) isozyme migrates toward the anode. Under these electrophoresis conditions, the human brain (BB) isoenzyme has already migrated off the top of the gel toward the anode.

electrophoresis (Figure 2) and compared the mobilities with human CK isozymes. We found that the electroplax cytosol contained two isoforms and that the major form comigrates with the *Torpedo* muscle enzyme. A second enzyme form was found to migrate to a position halfway between the brain and muscle enzyme. Electroplax membranes, purified for nicotinic receptor by sucrose gradient centrifugation, contained only muscle CK. The membrane-bound enzyme could also be demonstrated after extraction with alkali, the now standard treatment used to remove peripheral proteins from these membranes (Neubig et al., 1979), including ν_1. These isoenzyme electrophoresis experiments, besides clearly identifying the brain and muscle forms in *Torpedo*, also indicate that substantial differences between mammalian and elasmobranch CK exist. Under the conditions used (pH 8.6), the human BB enzyme migrates off the gel toward the anode, whereas the human MM form remains at the origin. The hybrid MB isozyme has intermediate mobility and migrates to the end of the gel toward the anode. In contrast, the relative mobilities of the *Torpedo* enzymes are reversed, with the brain enzyme remaining at the origin or migrating slightly toward the cathode, whereas the enzyme from *Torpedo* muscle and the electric organ migrates to a position halfway between the human MM and MB forms.

Earlier studies have indicated substantial differences in electrophoretic properties and tissue distribution in CK from lower vertebrates in comparison with mammalian or avian enzymes. After the tissue distribution of three fish CK isoenzymes that could not be classified according to localization in muscle and brain was analyzed, it was suggested that a nomenclature different from the traditional MM and BB

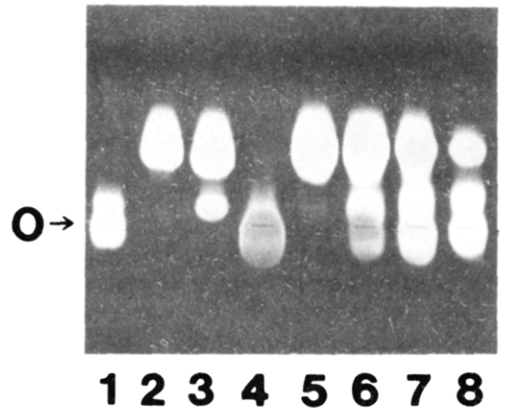

FIGURE 3: Hybridization of CK isozymes. The enzyme of intermediate mobility between brain and muscle found in electroplax cytosol (Figure 2, lane 1) could represent the *Torpedo* hybrid (MB) isozyme. To evaluate this possibility, the dimers were dissociated in urea and then allowed to reassociate in various combinations after removal of urea through dialysis. The result shows that a hybrid enzyme of the expected intermediate mobility does form under these conditions. Control samples in lanes 1–3 were from cytosol fractions that were dialyzed without the addition of urea. The samples in lanes 4–8 were brought to 8 M in urea and then dialyzed for 24 h against 0.1 M Tris buffer, pH 7.4, with 0.1 M 2-mercaptoethanol (Wevers et al., 1977). Isoenzyme analysis was then carried out as in Figure 2. Lanes 1 (brain), 2 (muscle), and 3 (electroplax) show the untreated cytosol. Lanes 4 (brain), 5 (muscle), and 6 (electroplax) are the corresponding samples after urea treatment. Brain and muscle cytosol were mixed prior to the urea treatment for the sample in lane 7, and brain and electroplax were mixed for lane 8.

designations be used (A_2, B_2, C_2; Fisher & Whitt, 1979). Our results suggest that, as is observed in higher vertebrates, only a single major form exists in brain and muscle of *Torpedo*. However, marked differences exist between *Torpedo* and mammalian or avian brain and muscle enzymes. This suggests that antibodies that are specific for chicken isoforms may not necessarily be useful for identifying homologous isozymes in *Torpedo*.

Hybridization of Brain and Muscle CK. The additional enzyme form that was found exclusively in electroplax cytosol is of intermediate mobility between the brain and muscle isozyme on agarose electrophoresis plates. In order to clarify the complexity of the different CK forms in this tissue, it is of interest to determine whether this isozyme consists of a unique third subunit or is a hybrid form consisting of brain and muscle subunits, as could be expected from its relative mobility. Evidence for the latter explanation is provided by a hybridization experiment performed as described by Wevers et al. (1977) for human CK. The native dimers are dissociated in 8 M urea and then allowed to randomly reassociate by removing the urea through dialysis. The enzymatic activity is restored to a significant extent during renaturation, and by a mixing of the CK fractions from different sources, the subunit composition of the original and reconstituted enzyme species may be deduced from their electrophoretic properties (Figure 3). As expected, the combination of brain and muscle cytosols generated a new form of intermediate mobility, comigrating with the previously observed intermediate form in electroplax cytosol. In addition, treatment of electroplax cytosol alone also gives rise to the brain form, which is not originally present in this fraction. This prompts the conclusion that the enzyme of intermediate mobility is most likely a brain/muscle heterodimer, since the brain form could not otherwise be generated. The limited resolution of the isozyme electrophoresis hinders the definitive identification of the intermediate enzyme as the heterodimer; however, evidence supporting this conclusion comes from antiserum overlays of

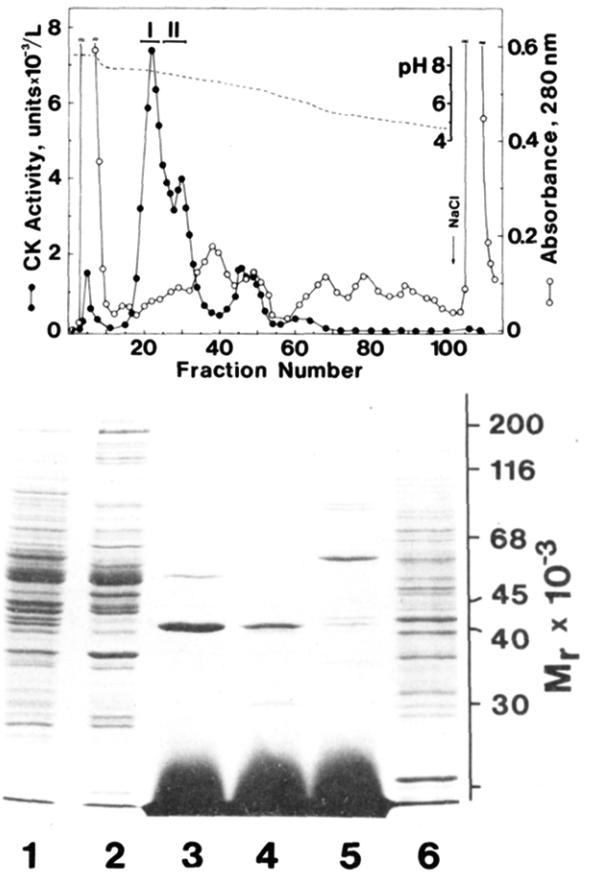

FIGURE 4: Chromatofocusing for the purification of *Torpedo* brain CK. Dialyzed cytosol, containing 117.5 mg of protein and 564 units of CK activity in 14.8 mL, was loaded on a chromatofocusing column (1.5 × 12.5 cm) that had been equilibrated in 25 mM Tris-HCl, pH 8.5. Elution was then started with 230 mL of Polybuffer of pH 6.5, followed by 260 mL of Polybuffer of pH 5.0. Fractions of 5.2 mL were collected at a flow rate of 11 mL/h. The diagram (top) shows the pH gradient (– –), protein profile (○), and CK activity (●). The double peak containing the bulk of CK activity was combined (pool I, fractions 19–24; pool II, fractions 25–31) for dialysis and concentration as described in the text and Table I. An arrow marks the start of elution with 1 M NaCl at the end of the pH gradient. The bottom panel shows the Coomassie profile of representative fractions after SDS gel electrophoresis: (lane 1) starting material (30 μg of protein); (lane 2) unadsorbed flow through (10 μL of fraction 5); (lane 3) main CK peak, part I (100 μL of fraction 21); (lane 4) main CK peak, part II (100 μL of fraction 28); (lane 5) second CK peak (100 μL of fraction 47); (lane 6) NaCl eluant (10 μL of dialyzed fraction 106).

isoenzyme gels where the intermediate form was found to react with the muscle-specific antiserum (data not shown).

Purification of Torpedo Brain CK. In order to further address the possibility that the brain CK is associated with receptor-enriched membranes, we have purified and identified CK from *Torpedo* brain. On the basis of our previous experience in purifying CK from electroplax, we used chromatofocusing as the first step (Figure 4). The cytosol from whole brain was dialyzed against the starting buffer of pH 8.5, and the precipitate that formed during dialysis was removed by centrifugation. The precipitated protein contains very little CK activity; thus, a slight increase in specific activity was observed (Table I). After the chromatofocusing column was loaded, a two-step elution was performed with buffers yielding a pH gradient from 8.5 to 5.0. We had previously determined that extending the gradient to pH 3.8 did not elute additional enzyme activity. Overall, 85% of the CK activity loaded on the column was recovered. Of the recovered activity, only 3% was contained in the large amount of unadsorbed protein,

Table I: Purification of *Torpedo* Brain CK by Chromatofocusing[a]

fraction	protein mg	protein %	CK activity units	CK activity units/mg of protein	CK activity %
cytosol	133.6	100	597	4.47	100
cytosol after dialysis	117.5	87.9	564	4.80	94.5
chromatofocusing					
pool I[b]	0.77	0.58	162	211	27.1
pool II[c]	1.44	1.08	100	69	16.8

[a] Three whole brains, weighing a total of 16.7 g, were homogenized and centrifuged as described under Experimental Procedures. The high-speed supernatant (cytosol) was dialyzed against the chromatofocusing loading buffer, precipitated protein was removed by centrifugation, and the supernatant was loaded on a chromatofocusing column and eluted as described in Figure 4. The fractions containing the double peak of CK activity were pooled as indicated in Figure 4 and the Polybuffer components removed by dialysis and concentrated with an Amicon YM-30 membrane. [b] Fractions 19–24 (Figure 4), eluting from pH 7.79 to 7.64. [c] Fractions 25–31, eluting from pH 7.58 to pH 7.40.

whereas 66% of the activity eluted in a double peak between pH 7.8 and pH 7.4. We had previously reported the electroplax enzyme ν_2 to elute at pH 6.6–6.9 (Gysin et al., 1983); since elution in chromatofocusing primarily is determined by a protein's p*I*, we conclude that the major brain enzyme is considerably more basic than the electroplax CK. A minor second peak, containing 13% of the activity, eluted at pH 7.0–6.6.

SDS electrophoresis of the CK-containing fractions showed that the enzyme activity was most likely associated with a protein band of 41 000 daltons (Figure 4, lanes 3 and 4), whereas most of the protein either was not adsorbed to the chromatofocusing column or was eluted with NaCl after completion of the pH gradient. The early and late fractions from the main peak were pooled separately, as indicated in Figure 4. After ultrafiltration and concentration in order to remove the Polybuffer components, which interfere with protein determinations and are visible as streaks near the dye front, a final purification of 47-fold was achieved at a specific activity of 211 units/mg of protein in pool I. The enzyme was estimated to be at least 80% pure on SDS gels. Minor contaminants could be removed through adsorption on Affi-Gel Blue and subsequent elution of CK with a gradient of 0–1 M NaCl. SDS gel electrophoresis shows a single band migrating at 41 000 daltons, slightly ahead of the 43 000-dalton electroplax protein (Figure 5). As reported by Barrantes et al. (1985), the membrane-associated CK, putatively identified as a brain enzyme on the basis of cross-reactivity with anti-chicken B-CK antiserum, was slightly higher in molecular weight and more acidic in p*I* than the electroplax enzyme from the cytoplasm (Barrantes et al., 1985). On the basis of these different molecular properties, we can definitively conclude that the acidic protein described by Barrantes et al. is not *Torpedo* brain CK.

Brain CK Is Absent from Electroplax Membranes. The pH at elution from a chromatofocusing column indicated that brain CK is considerably more basic than the muscle-type enzyme previously purified from electroplax. Combined with the lower molecular weight obtained from SDS electrophoresis, it can be expected that brain CK can be easily distinguished from the previously described 43 000-dalton or ν proteins. Two-dimensional gel electrophoresis confirms this molecular distinction between brain CK and the ν proteins (Figure 6). Two major spots, of approximate p*I* of 7.9 and 7.5, can be observed. When mixed with AChR-enriched membranes from electroplax, the two isoelectric brain CK variants are shown to be slightly more basic than ν_{1a} and ν_{1b}, the most basic

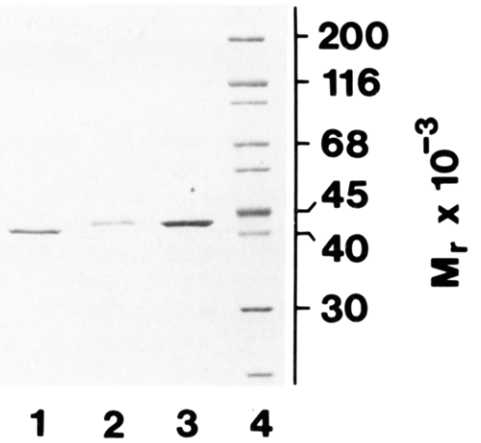

FIGURE 5: Molecular weight comparison of brain and electroplax CK. The molecular weight of *Torpedo* brain CK, determined from the relative mobility on 10% polyacrylamide–SDS gels, is slightly lower (41 000, lane 1) than that of the muscle-type enzyme purified from electroplax (43 000, lane 2). For comparison, rabbit muscle CK is also shown (43 000, lane 3). Proteins used as molecular weight standards are shown in lane 4.

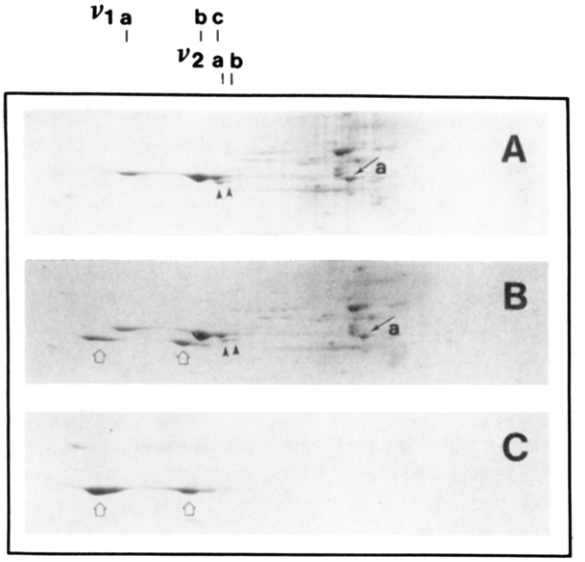

FIGURE 6: Two-dimensional gel electrophoresis of electroplax membranes and brain CK. In order to determine the position of brain CK in relation to the previously described proteins of *Torpedo* electroplax membranes, two-dimensional gel electrophoresis was performed (Gysin et al., 1981). Panel A contains 150 μg of AChR-rich membranes, purified by sucrose density gradient centrifugation. Panel B shows AChR membranes identical with those in (A), with the addition of 10 μg of brain CK (pool I from chromatofocusing). Panel C contains brain CK only. The positions of the main isoelectric focusing variants of ν_1 and the muscle-type CK ν_2, only visible in panels A and B, are indicated on top. Solid arrowheads indicate the position of ν_2 (panels A and B), whereas the main isoforms of B-CK are marked with open arrows (panels B and C). The arrow labeled a designates actin. The basic end of the isoelectric focusing dimension is to the left. Only the center portion of the gels containing the relevant molecular weight region is shown; this molecular weight region was chosen to include all visible minor spots in the highly purified brain CK fraction.

subunits of ν_1. The membranes alone, even at this relatively crude stage in the membrane purification procedure after sucrose density gradient centrifugation, do not contain the brain CK protein. This confirms the results obtained from isoenzyme analysis described above.

Discussion

The similarity of the electroplax CK with the muscle isoenzyme is supported by two lines of experimentation: im-

munological evidence and isozyme electrophoresis on agarose plates. Antiserum against electroplax CK reacts strongly with cytosol and particulate fractions from muscle but shows no reaction with *Torpedo* brain. The brain and muscle isozymes are therefore immunologically distinct. This is the case for other species where, despite extensive sequence homology between B and M isozymes (Pickering et al., 1985), polyclonal antisera are frequently specific for the individual isozymes (Eppenberger et al., 1967; Roberts et al., 1976; Armstrong et al., 1977; Perriard et al., 1978).

Isozyme electrophoresis confirms the identification of the electroplax CK, previously named ν_2 (Gysin et al., 1981, 1983), as the M form. In order to separate the B and M isozymes, running time and voltage had to be increased significantly as compared to the standard conditions used for separation of mammalian isozymes. Despite cooling, considerable spreading of the spots occurred after visualization, which degraded resolution somewhat. The mobility of the *Torpedo* M-CK is halfway between the sample origin and the position of mammalian MB, whereas *Torpedo* B-CK stays at the origin. Only a single form is displayed in brain and muscle cytosol; it is therefore practical to apply the convenient M and B nomenclature, despite the fact that the situation appears to be more complicated in other fishes (Champion et al., 1975; Fisher & Whitt, 1979). The electroplax cytosol contains two isoforms, the most prominent being MM, and a smaller amount of an intermediate form, most likely the MB hybrid. However, AChR-enriched membranes contain exclusively the MM form, as can be shown from analysis of sucrose gradient purified membranes and the alkaline extract prepared from these membranes or also membranes of higher purity prepared by affinity partitioning (Flanagan et al., 1976; Johansson et al., 1981). Hybridization experiments support the identification of the intermediate electroplax cytosol form as the MB hybrid. A new spot of identical mobility appears after mixing and hybridization of brain and muscle cytosol. Also, the form remaining at the origin, not previously detectable in electroplax cytosol, is generated during dissociation in urea and subsequent reassociation, compatible with the concept of random association of the individual M and B chains. The origin of the B chain in electroplax has not been established. It is conceivable that it stems from neuronal elements present in the densely innervated tissue where it would be most likely to occur in the BB form. Homogenization of the tissue could subsequently lead to a selective association of the B chains with M chains; this process may possibly be enhanced by freezing of the cytosol before isozyme analysis [see references in Watts (1973)], which is routinely done in our laboratory. This does not, however, explain the absence of the BB isozyme in electroplax. The possibility that the BB-CK activity may be selectively lost because it is much more labile than the MM and MB isozymes is unlikely, since we have not experienced more loss of enzyme activity during purification of the brain CK than that which occurred during purification of the MM-CK from electroplax.

An alternative possibility is that B chains are synthesized at a low level in the electrocytes. It has been well established that undifferentiated muscle cells synthesize primarily the B isozyme and that the switch to synthesis of the M form, such as it is exclusively found in adult muscle, is one of the early steps of muscle cell differentiation (Perriard et al., 1978; Caravatti et al., 1979; Dym & Yaffe, 1979). Morphological and biochemical transitions closely resembling those in myocytes occur in electrocytes during embryogenesis (Fox & Richardson, 1978; Krenz et al., 1980). Increased synthesis of mRNA for an M_r 43000 protein, identified as ν_2 at the time and therefore M-CK, was also described to occur during later embryonic stages (Witzemann et al., 1983). The *Torpedo* B-CK described here had not been previously identified, and its synthesis was therefore not followed during development. Despite extensive biochemical similarities between muscle and electric organ development, fundamental differences nevertheless exist. In particular, it has been described that an early myogenic stage of the electric organ is subsequently followed by an electrogenic stage (Fox & Richardson, 1979), during which the myofibrils disappear. This process has been called dedifferentiation on morphological grounds (Mellinger et al., 1978). It is therefore reasonable to speculate that the electric organ does not completely shut off the B-CK chain gene as is observed in adult muscle. Partial synthesis of B-CK could always be maintained at some level during development or turned off and then reexpressed later in a process that would be akin to biochemical dedifferentiation.

Before purification of the brain enzyme, we investigated individual brain areas for CK activity and isozyme patterns and found no regional differences in these parameters. Whole brain was therefore used as starting material with an initial specific activity of 4–6 units/mg in the cytosol, which is approximately 10-fold lower than the activity in electroplax cytosol (Gysin et al., 1983). The good recovery and high degree of purification that was obtained in a single step through chromatofocusing was similar to our experience with the electroplax CK. These results, together with a purification procedure described for human mitochondrial CK (Roberts & Grace, 1980; Grace et al., 1983), indicate that chromatofocusing can be applied in the purification of CK from various sources. The activity was found to be associated with a 41000-dalton protein band. Impurities could be removed by affinity chromatography on Affi-Gel Blue; however, due to the generally low recovery under various conditions tested, this step cannot be recommended for routine purifications.

As could be expected from the behavior during isoenzyme electrophoresis and from the elution position at pH 7.4–7.8 during chromatofocusing, B-CK was also considerably more basic than M-CK on two-dimensional gels. This is strikingly different from the situation in birds and mammals, where the brain enzyme is always more acidic than the muscle isoform. Recent sequence data have identified 14 additional lysines in M-CK when compared to the otherwise closely homologous B-CK in rabbit (Pickering et al., 1985). B-CK from *Torpedo* is therefore likely to show less sequence homology to B-CK of higher vertebrates, whereas the close homology of *Torpedo* M-CK or ν_2 with other species has already been established (Giraudat et al., 1984; West et al., 1984).

Our results fail to confirm the existence of *Torpedo* B-CK in AChR-rich membranes. The CK reactive with isozyme-specific anti-chicken B-CK antibody (Barrantes et al., 1983a,b, 1985; Wallimann et al., 1985) is considerably more acidic and of slightly higher molecular weight than M-CK. These characteristics are distinctly different from the B enzyme described in this paper. It has been suggested that the affinity purification procedure used in our laboratory for the preparation of highly purified AChR membranes may be responsible for this discrepancy (Barrantes et al., 1983b). However, in membranes that have been prepared through conventional sucrose gradient centrifugation, B-CK is absent during isozyme electrophoresis (Figure 2, lane 4) and on two-dimensional gels (Figure 6A). Also, no mammalian-like B-CK was detected in these experiments. One possible explanation is that the activity described by Barrantes et al. is much more labile than

that of the other isozymes found in the electroplax and is therefore rapidly inactivated during the membrane purification procedures. While this seems unlikely from the accounts of the purification of the CK activity reported by Barrantes et al. (1985), this possibility cannot be completely ruled out.

While this work was being prepared for publication, two reports have appeared with results largely similar to the ones described (Perryman et al., 1985; Witzemann, 1985). Both reports confirm the electroplax CK as the muscle isozyme and did not detect B-CK in the electroplax tissue. Also, similar to our findings in *Torpedo californica*, the brain enzyme described by Witzemann (1985), purified from *Torpedo marmorata*, was found to be more basic (p*I* = 7.0–7.2) and of lower molecular mass (42000) than the electroplax and muscle CK. This makes it highly unlikely that species differences exist in the CK isozyme patterns of the *Torpedo* family that could explain the finding of B-CK in electroplax membranes.

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Factors Affecting the Activation of Rabbit Muscle Phosphofructokinase by Actin†

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ABSTRACT: The consistent application of phosphatase inhibitors and a novel final purification step using a connected series of DE-51, DE-52, and DE-53 anion-exchange chromatography columns facilitate the preparation of electrophoretically homogeneous subpopulations of rabbit muscle phosphofructokinase which differ in their catalytic properties and endogenous covalent phosphate content. A band of "high"-phosphate enzyme (fraction II) flanked by regions of "low"-phosphate enzyme (fractions I and III) is an unusual feature of the final purification profile. Fractions I (containing in this case 0.42 mol of P/82 000 g of enzyme) and II (containing 1.26 mol of P/82 000 g of enzyme) exhibit the most pronounced functional differences of the fractions. Following our original report [Liou, R.-S., & Anderson, S. R. (1980) *Biochemistry 19*, 2684], both are activated by the addition of rabbit skeletal muscle F-actin. Under the assay conditions, half-maximal stimulation of phosphofructokinase activity occurs at 15.4 nM actin (in terms of monomer) for fraction I and 9.7 nM for fraction II. The low-phosphate enzyme is synergistically activated in the presence of 0.12 μM actin plus 3.0 μM fructose 2,6-bisphosphate, with a marked increase in V_{max}, while the high-phosphate enzyme is not. Neither fraction is activated appreciably by the addition of G-actin or the chymotrypsin-resistant actin "core". The covalently cross-linked trimer of actin stimulates the activity of both the low- and high-phosphate enzyme fractions. However, the previously mentioned synergistic activation characteristic of fraction I fails to occur in solutions containing the trimer plus fructose 2,6-bisphosphate. Phosphorylation of fraction I in an in vitro reaction catalyzed by the cAMP-dependent protein kinase causes its properties to become more like those of fraction II. The total amount of covalent phosphate present after in vitro phosphorylation approaches 2 mol of P/82 000 g of enzyme for both fractions.

The enzyme 6-phosphofructo-1-kinase (phosphofructokinase) (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is considered to be the controlling enzyme in the glycolytic pathway of yeast and mammals, catalyzing the phosphorylation of fructose 6-phosphate by Mg-ATP to give fructose 1,6-bisphosphate and Mg-ADP [cf. review by Hofmann (1978)]. As such, the enzyme is subject to close regulation by a number of allosteric effectors, including inhibitors such as nucleotide triphosphates and citrate and positive effectors such as glucose 1,6-bisphosphate and fructose 1,6-bisphosphate [cf. reviews by Kemp & Foe (1983), Uyeda (1979), and Goldhammer & Paradies (1979)]. A novel sugar phosphate, fructose 2,6-bisphosphate, is a potent activator of phosphofructokinase and is synthesized from and degraded to fructose 6-phosphate by a specific kinase and phosphatase, respectively. The intracellular levels of fructose 2,6-bisphosphate are influenced by a variety of hormonal and metabolic factors which also affect the activity of phosphofructokinase. The synthesis and degradation of fructose 2,6-bisphosphate by a specific kinase and phosphatase are directly controlled by the level of cyclic AMP via the cAMP-dependent protein kinase [cf. reviews by Hers & Van Schaftingen (1982), Pilkis et al. (1982), Furuya et al. (1982), and Hers & Hue (1983)].

Accumulating evidence shows that macromolecular interactions also affect the catalytic activity of the enzyme. In the case of rabbit muscle phosphofructokinase, experiments have shown that the catalytic activity (Hofer, 1970; Lad et al., 1973) and substrate binding (Hill & Hammes, 1975) are both affected by the self-association of the enzyme. Although the tetramer appears to be the smallest active species of the enzyme, the self-associated forms may play some role in the regulation (Luther et al., 1983).

Like several other glycolytic enzymes, phosphofructokinase can be covalently modified by phosphorylation, suggesting the existence of a specific protein kinase and phosphatase. Initial reports of phosphorylation applied to liver extracts (Brand & Soling, 1975). Hofer and Furst (1976) later isolated ^{32}P-labeled phosphofructokinase from the skeletal muscle of mice that had been injected with [^{32}P]P_i. However, phosphorylation of rabbit muscle phosphofructokinase has little effect upon enzyme activity [cf. reviews by Söling & Brand (1981), Clark

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