Cyclic Adenosine 3':5'-Monophosphate-dependent Protein Kinase

COMPARISON OF TYPE II ENZYMES FROM BOVINE BRAIN, SKELETAL MUSCLE, AND CARDIAC MUSCLE*

F. Thomas Hartl‡ and Robert Roskoski, Jr.

From the Department of Biochemistry, Louisiana State University Medical Center, New Orleans, Louisiana 70112

The physical and chemical properties of purified catalytic and regulatory subunits of type II cAMP-dependent protein kinase from bovine brain, skeletal muscle, and cardiac muscle were compared. The catalytic subunits from all three sources were identical with respect to molecular weight \( (M_w = 40,000) \), amino acid composition, and isoelectric points. Furthermore, two-dimensional maps of their tryptic peptides were identical. The type II regulatory subunits from brain and skeletal muscle exhibited identical molecular weights by sodium dodecyl sulfate-gel electrophoresis and both undergo autophosphorylation; however, they differ somewhat in amino acid composition. Furthermore, two-dimensional tryptic peptide maps showed that 40% of the peptides differ and 60% co-migrate. Autoradiography of the peptide maps showed that the main phosphorlated peptide from these two sources also differ. The bovine brain type II regulatory subunit also differed from that of bovine cardiac muscle in the same manner. The molecular weights, amino acid composition, and tryptic peptide maps of the bovine skeletal and cardiac muscle regulatory subunits, however, were experimentally identical. These results suggest that type II protein kinases from bovine brain and muscle (skeletal or cardiac) represent distinct species which differ in their regulatory subunits but share a common catalytic subunit.

The tryptic peptide map of the type I regulatory subunit from bovine skeletal muscle was very different from that of the two classes of type II regulatory subunit. There were, however, four polar peptides from neural and nonneural type II subunits as well as the type I regulatory subunit which co-migrated.

Adenosine 3':5'-monophosphate-dependent protein kinases (ATP:protein phosphotransferase; EC 2.7.1.37) play an important regulatory role in many metabolic and physiological processes. These enzymes mediate the effects of cAMP through phosphorylation of specific proteins (cf. Refs. 1-4). In the cell, the enzyme exists as an inactive tetrameric holoenzyme consisting of dissimilar regulatory (R) and catalytic (C) subunits, which is activated by cAMP according to the equation (15, 36):

\[ R_4C_4 + 4cAMP \rightleftharpoons R_4(C_cAMP)_4 + 2C \]

At least two forms of the enzyme which can be separated by ion-exchange chromatography occur in cells. These have been termed type I and type II on the basis of their order of elution from DEAE-cellulose (5, 6). Their regulatory subunits differ in molecular weight (7,8), amino acid composition (8,15), and immunological determinants (9). Furthermore, the tryptic peptide maps of type I and type II regulatory subunits from porcine skeletal muscle exhibit little similarity, which suggests that their primary structures differ (8). These dissimilarities result in holoenzymes with different properties. Type I holoenzyme from rabbit skeletal muscle, for example, possesses a high affinity MgATP-binding site (60 nM) which is not demonstrable in the isolated subunits (7,10). Furthermore, the type II enzymes from bovine cardiac muscle (11) and brain (12) undergo an intramolecular autophosphorylation.

The type II cAMP-dependent protein kinase from bovine cardiac muscle is the best characterized form of the enzyme. The complete amino acid sequences of the catalytic (13) and regulatory (14) subunits have been determined. Much less, however, is known about the type II enzyme from brain, although some of the physicochemical properties of this enzyme were studied in crude preparations from bovine cerebral cortex by Rubin and co-workers (17). They found that the brain enzyme resembles the heart enzyme in holoenzyme molecular weight, subunit molecular weights, ability to undergo autophosphorylation, and isolectric points of 8-azido-[\(^32\)P]cAMP-labeled regulatory subunits in 8 M urea. However, the regulatory subunits from enzymes differ immunochemically. This has been determined using specific antisera elicited by the enzymes from bovine cardiac muscle (9,17) and bovine brain (18). On this basis, it has been suggested that type II protein kinases from neural and nonneural sources represent related but distinct subclasses of type II protein kinases (17,18).

To further characterize the differences between neural and nonneural type II protein kinases, we have compared purified regulatory and catalytic subunits from bovine brain, skeletal muscle, and cardiac muscle using a variety of techniques. Although the type II regulatory subunit from brain is similar in molecular weight and amino acid composition to the type II regulatory subunits from skeletal muscle, the two-dimensional maps of its tryptic peptides differ from those of the skeletal or cardiac muscle regulatory subunits. In contrast, the catalytic subunits from all three sources were identical with respect to molecular weight, amino acid composition, isolectric points, and two-dimensional tryptic peptide maps. Our results suggest that the type II protein kinases from bovine brain and muscle (cardiac or skeletal) represent unique species which share a common catalytic subunit but differ in their regulatory subunits.

* This work was supported by Grant NS-15994 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address, Department of Medicine, Columbia University College of Physicians and Surgeons, 639 West 168th Street, New York, NY 10032.

(Received for publication, October 7, 1982)
Comparison of Type II Protein Kinases

**EXPERIMENTAL PROCEDURES**

Catalytic subunits and regulatory subunits from bovine brain were purified as described (20). Components were purified from skeletal muscle using the same methodology. Catalytic and regulatory subunits from bovine cardiac muscle were a generous gift from Dr. Edwin G. Krebs (University of Washington, Seattle, WA). Trypsin-L-1-tosylamido-2-phenylethyl chloromethyl ketone was obtained from Worthington.

**Protein Kinase Assays**—Protein kinase activity was measured using the phosphocellulose adsorption procedure of Witt and Roskoski (21) using Ser-peptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as substrate and washing the phosphocellulose paper strips in 75 mM H3PO4, 0.1 M NaCl, 0.1 M Tris-HCl (pH 6.9), and 25 mM EDTA. Ser-peptide was isolated from bovine cardiac muscle using the same methodology. Catalytic and regulatory subunits were compared by several methods. Some of these results are summarized in Table I.

**Amino Acid Analysis**—Analyses were performed on an automated Beckman analyzer using a single column system. Hydrolyses were performed in vacuo at 110 °C for 24, 48, and 72 h in 6 N HCl. Amino-β-guanidinopropionic acid (Pierce Chemical Co.) was added to all samples as an internal standard. Values for serine, threonine, and tyrosine were calculated by extrapolating to zero time of hydrolysis. Tryptophan was determined by its fluorescence in 6 M guanidine hydrochloride as described by Pajot (25).

**Phosphorylation of Regulatory Subunits**—Type II regulatory subunits from bovine brain, skeletal muscle, or cardiac muscle (2 mg) were incubated at 0 °C in a reaction mix containing 50 mM sodium phosphate (pH 7.0), 0.1 mM NaCl, 10 mM MgSO4, 0.1 M β-glycerol phosphate, and 1 mM ATP. The reaction was initiated by the addition of 15 μg of catalytic subunit. After 15 min, the reaction mixture was chromatographed on a column (1 × 15 cm) of Sephadex G-50-80 equilibrated with 50 mM ammonium bicarbonate (pH 8.3).

**Tryptic Digestion**—Before digestion, samples were carboxymethylated by incubation with 2.5 mM iodoacetic acid (Sigma) at room temperature for 1 h. The samples were then dialyzed exhaustively against 40 mM ammonium bicarbonate (pH 8.3). To facilitate digestion, the protein samples were first denatured by heating in a boiling water bath for 10 min in a tightly stopped tube. In some cases (notably the catalytic subunits), a white precipitate formed upon heating. When this occurred, the sample was sonicated to yield a very finely dispersed precipitate. Trypsin-L-1-tosylamido-2-phenylethyl chloromethyl ketone (Worthington) was added (10 μg/mg of protein kinase), and the digestion was carried out at 37 °C for 7-13 h. In those cases where a precipitate had formed upon heating, digestion was accompanied by a loss of turbidity. The digests were lyophilized over solid NaOH (to aid in removal of ammonium bicarbonate). The residue was redissolved in H2O and relyophilized; this process was repeated four times. The lyophilized digests were stored at −20 °C until needed.

**Sample Preparation**—Tryptic digests were dissolved in electrophoresis buffer (pyridine-acetic acid-H2O: 5:5:390; pH 4.7) at an approximate concentration of 10-20 mg/ml and centrifuged for 5 min at 13,600 × g in an Eppendorf microfuge to remove insoluble material. These solutions were then used for two-dimensional peptide mapping.

**Peptide Mapping**—Two-dimensional peptide maps were prepared on plastic-backed cellulose thin layer plates (20 × 20 cm, 0.1-mm thick, EM Reagents). After application of samples (0.1-0.2 mg in a 5-mm line), the plates were wetted with acetic acid:pyridine:H2O (5:5:390, pH 4.7) and electrophoresed for 90 min (catalytic subunits) or 100 min (regulatory subunits) at 500 V in this same buffer. The plates were air-dried in a hood for 1 h and then developed in the second dimension by ascending chromatography using 1-butanol-acetic acid:pyridine:H2O (14:3:5:7, v/v). The plates were air-dried in the hood and stained with cadium ninhydrin (27).

**RESULTS**

To characterize the type II protein kinases from bovine brain, heart, and skeletal muscle, purified catalytic and regulatory subunits were compared by several methods. Some of these results are summarized in Table I.

**Comparison of Catalytic Subunits**—The catalytic subunits from all three tissues were indistinguishable by these methods. When subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, all migrated as single bands with molecular weights of 40,000 (Fig. 1). On isoelectric focusing, each showed two major bands with isoelectric points at 7.05 and 7.45 (Fig. 2). Furthermore, the amino acid compositions of the three catalytic subunits were, within experi-

![Fig. 1. Polyacrylamide gel electrophoresis of purified catalytic and regulatory subunits. Samples (10 μg) of type II regulatory subunits from skeletal muscle (MR1) and brain (BR1), type I regulatory subunit from skeletal muscle (MR1), and catalytic subunit from skeletal muscle (MC) and brain (BC) were electrophoresed in a 10% polyacrylamide slab gel using a Tris glycine buffer system (22) and stained with Coomassie blue (R-250) as described under "Experimental Procedures."](image1)

![Fig. 2. Isoelectric focusing of type II catalytic subunits. Samples of type II catalytic subunit (10 μg) were electrophoresed in 5% polyacrylamide gels (Ampholine PAG plates, pH 5.0-8.5) using an LKB 2117 Multiphor apparatus. After allowing the pH gradient to form for 45 min, the samples were applied to the gels and focused for 2 h at 4°C. Lane A, cardiac muscle catalytic subunit; lane B, skeletal muscle catalytic subunit; lane C, brain catalytic subunit.](image2)
Comparison of Type II Protein Kinases

Amino acid compositions of type II catalytic and regulatory subunits from bovine brain, skeletal muscle, and cardiac muscle

<table>
<thead>
<tr>
<th></th>
<th>Catalytic subunit</th>
<th>Regulatory subunit</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Brain</td>
<td>Muscle</td>
<td>Heart</td>
<td>Brain</td>
<td>Muscle</td>
</tr>
<tr>
<td>Arg</td>
<td>15.8</td>
<td>14.9</td>
<td>15</td>
<td>26.1</td>
<td>27.1</td>
</tr>
<tr>
<td>Asp</td>
<td>38.8</td>
<td>36.8</td>
<td>35</td>
<td>47.6</td>
<td>47.2</td>
</tr>
<tr>
<td>Asx</td>
<td>38.8</td>
<td>36.8</td>
<td>35</td>
<td>47.6</td>
<td>47.2</td>
</tr>
<tr>
<td>Glu</td>
<td>41.5</td>
<td>41.6</td>
<td>41</td>
<td>62.5</td>
<td>56.9</td>
</tr>
<tr>
<td>Gly</td>
<td>21.2</td>
<td>22.5</td>
<td>22</td>
<td>32.4</td>
<td>26.6</td>
</tr>
<tr>
<td>His</td>
<td>8.9</td>
<td>8.6</td>
<td>9</td>
<td>19.5</td>
<td>20.4</td>
</tr>
<tr>
<td>Ile</td>
<td>18.5</td>
<td>18.6</td>
<td>21</td>
<td>31.0</td>
<td>30.4</td>
</tr>
<tr>
<td>Leu</td>
<td>30.8</td>
<td>33.1</td>
<td>32</td>
<td>82.1</td>
<td>11.1</td>
</tr>
<tr>
<td>Lys</td>
<td>32.8</td>
<td>35.3</td>
<td>34</td>
<td>20.2</td>
<td>21.6</td>
</tr>
<tr>
<td>Met</td>
<td>6.2</td>
<td>7.2</td>
<td>7</td>
<td>9.6</td>
<td>8.4</td>
</tr>
<tr>
<td>Phe</td>
<td>25.7</td>
<td>24.0</td>
<td>25</td>
<td>17.6</td>
<td>14.9</td>
</tr>
<tr>
<td>Pro</td>
<td>14.2</td>
<td>13.5</td>
<td>14</td>
<td>18.7</td>
<td>20.6</td>
</tr>
<tr>
<td>Ser</td>
<td>18.6</td>
<td>20.5</td>
<td>16</td>
<td>22.2</td>
<td>23.4</td>
</tr>
<tr>
<td>Thr</td>
<td>6.2</td>
<td>7.2</td>
<td>7</td>
<td>9.6</td>
<td>8.4</td>
</tr>
<tr>
<td>Thr</td>
<td>13.7</td>
<td>13.5</td>
<td>14</td>
<td>19.2</td>
<td>17.2</td>
</tr>
<tr>
<td>Trp</td>
<td>5.7</td>
<td>6.2</td>
<td>6</td>
<td>1.5</td>
<td>1.2</td>
</tr>
</tbody>
</table>

*From Shoji et al. (13).

*From Takio et al. (14).

*From a 72-h hydrolysis.

Table II shows significant differences from the type II regulatory subunits from bovine heart or skeletal muscle. All three forms migrate on sodium dodecyl sulfate-polyacrylamide gels as doublets of weight 55,000–57,000 (Fig. 1) presumably due to the presence of phospho and dephospho forms (8, 11, 17) which we have confirmed. Whereas the slower migrating (phospho) band is predominant in the skeletal muscle regulatory subunit, the faster migrating (dephospho) band was the predominant form observed in the regulatory subunit from brain. Since the skeletal muscle and brain regulatory subunits are prepared by identical methodologies, the brain may contain greater endogenous phosphatase activity.

Some minor differences were observed in the amino acid composition of the regulatory subunits from brain and skeletal or cardiac muscle (Table II). The greatest differences were observed in glycine, alanine, valine, and glutamic acid: glutamine with smaller differences in tyrosine and phenylalanine. The compositions are, however, remarkably similar. However, when the regulatory subunits were carboxymethylated and digested with trypsin and the resulting peptides were resolved in two dimensions, significant differences between the brain and muscle regulatory subunits became apparent. Using the methodology described under “Experimental Procedures,” approximately 50 peptides could be resolved from brain, heart, or skeletal muscle type II regulatory subunits. When mixtures of tryptic digests from heart and skeletal muscle regulatory subunits (which were purified in different laboratories by different procedures) were mapped together, the resulting pattern of 50 peptides was identical with the maps of the digests from the individual heart or skeletal muscle subunits (Fig. 4). This suggests that the regulatory subunits from cardiac and skeletal muscle are very similar, if not identical. In contrast, peptide maps of brain and skeletal muscle regulatory subunits were different (Fig. 5). When tryptic digests from each were combined, the resulting pattern contained approximately 65 ninhydrin-positive spots. Of these, 15 were unique to brain regulatory subunit, 15 were unique to muscle regulatory subunit, and 30–35 were common to both subunits. This indicates an approximate 60% homology between the subunits. These results suggest that the type II regulatory subunits from brain and skeletal (or cardiac) muscle share many common sequences, but each contain sequences which are unique.

We further characterized the peptides which contain the autophosphorylated residue. Autoradiographs of the maps in Fig. 5 showed several 32P-containing peptides (Fig. 6) which...
migrated slowly during electrophoresis and poorly during chromatography, indicating that they were highly polar, acidic peptides. The patterns for brain and skeletal muscle regulatory subunits were similar but not identical. The reason for the heterogeneity in the phosphopeptides is not clear. This heterogeneity was observed when samples of regulatory subunits were incubated with trypsin (10 μg/mg of regulatory subunit) for 3, 7, 10, or 13 h at 37 °C. This may be due to contamination in our trypsin-L-1-tosylamido-2-phenylethyl chloromethyl ketone (e.g., residual chymotrypsin) or due to the intrinsic chymotryptic-like activity of trypsin. Alternatively, it may be due to some structural feature or features in the region of the autophosphorylation site. The sequence on the COOH-terminal side of this site in the heart type I1 regulatory subunit contains a stretch of 6 acidic residues and 2 prolines (14). This could form a structure which is somewhat resistant to trypsin, resulting in incomplete digestion. The observed heterogeneity could be due, inter alia, to one or all of these possibilities. The differences between the phosphopeptide patterns of the brain and muscle regulatory subunits
Comparison of Type II Protein Kinases

(Fig. 6) suggests that dissimilarities exist between the amino acid sequences in the regions of their autophosphorylation sites. To fully characterize this, however, would require that the peptides be purified and sequenced.

The type II holoenzymes from bovine brain and skeletal muscle showed different concentration dependencies for dissociation by cAMP. Type II holoenzyme from brain requires approximately a 10-fold higher concentration of cAMP for dissociation than does the type II enzyme from skeletal muscle (Fig. 7). This is consistent with the observation (18) that the brain regulatory subunit has a lower apparent affinity than the cardiac muscle subunit for cAMP. Hoppe and co-workers (19) reported that the $K_i$ of cAMP for the catalytic subunit is 200 nM. This is the probable explanation for the inhibition of phosphotransferase activity observed at high cAMP concentrations (Fig. 7).

The two-dimensional tryptic peptide maps from type II regulatory subunits from skeletal muscle and brain were compared with those of the type I regulatory subunit from skeletal muscle. Neither skeletal muscle (Fig. 8) nor brain (Fig. 9) type II regulatory subunits showed much homology with the type I regulatory subunit. However, some basic histidine peptides appear to co-migrate from all three subunits. Determination of the compositions and sequences of these peptides is necessary to determine if this represents a feature common to all three forms of regulatory subunit.

**DISCUSSION**

We have directly compared both catalytic and regulatory subunits from type II cAMP-dependent protein kinases from bovine heart, skeletal muscle, and brain. The catalytic subunits from these sources were indistinguishable by any of the techniques used in this study. They were identical with respect to molecular weight, amino acid composition, isoelectric points, and two-dimensional tryptic peptide maps. This is not surprising in view of the great similarities in catalytic subunits from different tissues and species previously reported in the literature. Catalytic subunits from protein kinases isolated from different tissues and species (either type I or type II) are very similar in molecular weight (7, 8, 29), substrate specificity (28), and kinetic parameters (7, 22). We have found no differences in the type II catalytic subunits from bovine brain, heart, or skeletal muscle, suggesting that the type II protein kinases from these different sources share a common catalytic subunit. However, Zoller and co-workers (8) compared the type I and type II enzymes isolated from porcine skeletal muscle and found subtle differences in the two-dimensional tryptic peptide maps of the catalytic subunits. This is one of the few differences between catalytic subunits which has been reported to date.

In contrast to the catalytic subunits, which were indistinguishable by the techniques we used, the type II regulatory subunits from brain and cardiac or skeletal muscle show similarities in some properties but significant differences in others. All showed apparent molecular weights of 55,000 on sodium dodecyl sulfate-polyacrylamide gels. The complete amino acid sequence of the type II regulatory subunit from bovine cardiac muscle has recently been elucidated (14). The actual molecular weight for this protein, calculated from the sequence, is 45,000 rather than the value of 54,000–57,000 observed here and reported elsewhere for type II regulatory subunits from neural (17) and nonneural (7, 8, 15, 30) sources. For this reason, the amino acid compositions of both the skeletal muscle and brain type II regulatory subunits were calculated based on the molecular weight corresponding to the sequence of the heart regulatory subunit rather than the molecular weight calculated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Although some differences were noted, the amino acid compositions of the brain and skeletal muscle subunits were remarkably similar to each other and to that of the cardiac muscle regulatory subunit (14).

Two-dimensional maps of the tryptic peptides from phosphorylated type II regulatory subunits from cardiac or skeletal muscle were virtually identical. However, the tryptic peptide maps of the type II regulatory subunit from brain were significantly different from the cardiac or skeletal muscle peptide maps, indicating sequence differences in the proteins. Approximately two-thirds of their peptides were superimposable, while each subunit contained 15–20 unique peptides which were absent from the other. This suggests that although there are regions of sequence homology between the two proteins, there exist regions in the two regulatory subunits which are distinctly different. By contrast, the tryptic peptide maps of type II regulatory subunits from brain or skeletal muscle showed very little similarity to those of type I regulatory subunit, suggesting that the type I and type II regulatory subunits differ significantly in primary structure. This agrees with the results of Zoller and co-workers (8) who compared type I and type II protein kinases from porcine skeletal muscle. These workers suggested that although the type I and type II regulatory subunits may differ in primary structure, they may have similar "core regions" structurally. This is supported by data which indicate that hybrid holoenzymes can be formed by mixing type I and type II catalytic and regulatory subunits (26, 28).

Differences in primary structure and resultant alterations in secondary and tertiary structures could account for the dissimilarities in antigenic determinants possessed by the brain and cardiac muscle enzymes (9, 17, 18). A series of monoclonal antibodies against bovine cardiac muscle type II regulatory subunit has been produced (32, 33). Some of these have been used as structural probes of type II regulatory subunits (33, 34). These could prove to be an important tool in studying the similarities and differences in type II regulatory subunits from neural and nonneural sources.

The brain and muscle type II regulatory subunits appear to differ in the region surrounding their autophosphorylation sites. Autoradiographs of the two-dimensional peptide maps of $^{32}$P-labeled regulatory subunits from brain and muscle (cardiac or skeletal) were dissimilar. Since the region surrounding the autophosphorylation site is thought to be involved in recognition of the catalytic subunit (14, 34), changes in this portion of the molecule could result in altered R-C interactions. We have observed differences in the interactions of the catalytic subunit with regulatory subunits from brain and skeletal muscle in the presence of cAMP (20). In the absence of CAMP, brain and muscle regulatory subunits protect the cysteiny l residues in the catalytic subunit against modification by sulphydryl group-specific chemical modifica tion reagents (20, 31). However, in the presence of saturating concentrations of CAMP, muscle type II regulatory subunit, but not brain regulatory subunit, accelerates the rate of modification of catalytic subunit sulphydryl groups (20, 31).

The results presented here are in full accordance with the hypothesis (17, 18) that type II protein kinases from neural and nonneural sources represent distinct but related subclasses of type II protein kinases. Furthermore, the enzymes of these subclasses differ in their regulatory subunits but share a common catalytic subunit. The ultimate determination of the extent of differences between the regulatory subunits will come about when the amino acid sequence of the brain protein is determined and compared with the known sequence of the cardiac muscle regulatory subunit.
One question raised by these studies is whether the type II regulatory subunits from neural and nonneural sources are the products of distinct (but similar) genes or whether they arise from the same gene via different mRNA processing or post-translational modification. Clearly, more information on the structure of neural protein kinases, as well as the structure of the protein kinase gene(s), is necessary to deduce the origin of multiple forms of cAMP-dependent protein kinase.

Acknowledgments—We would like to thank Dr. William T. Morgan for the use of his amino acid analyzer and Karen L. Vrana for secretarial assistance.

REFERENCES
16. Deleted in proof
23. Deleted in proof
24. Deleted in proof