Vol. 95, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 31, 1980 Pages 569-576

EVIDENCE THAT A 41,000 DALTON BRAIN PHOSPHOPROTEIN IS PYRUVATE DEHYDROGENASE

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Received June 6,1980

Summary: Phosphorylation of a brain protein of M_r=41,000, termed band F_2, is selectively regulated by effectors of pyruvate dehydrogenase kinase (pyruvate, dichloroacetate, NAD, NADH, CoA, and acetyl CoA). Subcellular fractionation studies indicate a mitochondrial localization of a phosphoprotein with this molecular weight. The phosphorylated α-subunit of purified bovine kidney pyruvate dehydrogenase comigrates with band F_2 on polyacrylamide gels and both appear as a doublet band of M_r=41,000-42,000. On the basis of similar regulatory properties, subcellular location and electrophoretic mobility, we propose that band F_2 is the α-subunit of the brain pyruvate dehydrogenase complex. Because band F_2 can be affected by physiological and behavioral treatments, our hypothesis suggests a potential regulatory role for pyruvate dehydrogenase in brain function.

In the previous report from this laboratory (1) a phosphoprotein with M_r=41,000 has been designated band F_2. The phosphorylation of a protein in this molecular weight range had been shown by several laboratories to be affected by treatments which alter neuronal activity. The in vitro phosphorylation of this band with $[^{32}P]$ATP is sensitive to injections of pentobarbital (1) or ACTH (2), chronic administration of opiates (3,4), high frequency electrical stimulation of the hippocampal slice (5), or acquisition of an aversively motivated learning task (6).

Converging lines of evidence now suggest that the $[^{32}P]$ containing band F_2 is the phosphorylated α-subunit of the pyruvate dehydrogenase complex in brain. This report contains the first evidence to support this hypothesis. We show here that compounds which regulate the phosphorylation of pyruvate dehydrogenase, selectively regulate the phosphorylation of band F_2 in the predicted direction (7).

Abbreviations used: M_r, apparent molecular weight; SDS, Sodium dodecyl sulfate.

0006-291X/80/140569-08$01.00/0

Materials and Methods

The protein source for these studies was the forebrain of male albino rats (Holtzman strain) sacrificed by whole body immersion in liquid nitrogen. The frozen brain minus brainstem and cerebellum was homogenized with a motor driven teflon pestle in 20 volumes of 30 mM potassium phosphate buffer (pH-7.2; 2°C). Aliquots were removed and frozen at -20°C until used in the phosphorylation assay.

[γ - ^{32}P] ATP (S.A.=160 Ci/mM) was purchased from I.C.N. (Irvine, CA.). Sodium ATP, sodium pyruvate, CoA-SH and acetyl CoA were obtained from Sigma (St. Louis, MO.). Dichloroacetic acid (Aldrich, Milwaukee, WI.) was neutralized with NaOH prior to use. Acrylamide and N,N′-methylenebisacrylamide were supplied by Polysciences (Warrington, PA.). Other polyacrylamide reagents were purchased from Eastman Organic Chemicals (Rochester, N.Y.). NAD , NADH and Coomassie Blue were obtained from Boehringer-Mannheim (Indianapolis, IND.).

Purified bovine kidney pyruvate dehydrogenase complex and α-subunit were the generous gift of Dr. Lester Reed, University of Texas, Austin, TX.

In vitro protein phosphorylation: The frozen brain homogenate material was preincubated for 3 minutes at 30°C and then reacted with 0.5 mM [^{32}P] ATP in 30 mM potassium phosphate buffer (pH 7.2), 1 mM Mg Cl_2, 0.1 mM EDTA, 2 mM dithiothreitol, plus effectors of pyruvate dehydrogenase phosphorylation (where indicated). Thirty seconds later 100 µl aliquots were solubilized in an SDS-electrophoresis solution (final concentrations: 3% SDS; 2% β-mercaptoethanol; 6% sucrose, 0.01% bromophenol blue; 10 mM Tris · Cl, pH-6.9; 5 mM EDTA). At the same time reaction aliquots were precipitated with 10% TCA (8) to determine the total [^{32}P] incorporation.

Polyacrylamide gel electrophoresis and autoradiography: Aliquots containing 50 µg of SDS-solubilized protein from the phosphorylation reaction were electrophoresed on 10% acrylamide, 0.27% bisacrylamide gels as described previously(6). Following electrophoresis, the gels were fixed in 10% acetic acid and stained for protein with Coomassie Blue. The gels were dried and exposed to Kodak No Screen X-ray film for several days. The X-ray film was developed by conventional methods to produce an autoradiogram.

Densitometric Analysis: Autoradiograms were scanned with an EC-Apparatus 920 microdensitormeter. The base and height of each peak were measured minus background density, and the area of each peak computed (A=bh/2). Peak height alone (6) does not take into account that the width and height of a peak can vary independently.

Protein estimation: Protein concentration of brain homogenates was estimated by the method of Lowry, et al. (9), using bovine serum albumin as standard.

Results

Two known inhibitors of pyruvate dehydrogenase kinase are dichloroacetic acid and the substrate of the pyruvate dehydrogenase complex, pyruvate (10,11). When either dichloroacetic acid or sodium pyruvate were included in the in vitro phosphorylation reaction with brain homogenates, a selective reduction in the [^{32}P] incorporation into band F_2 was observed. The autoradiogram in Figure 1 shows the protein phosphorylation pattern obtained in the presence of 1 mM or 5 mM dichloroacetate (lanes 2 and 3, respectively) or 5 mM sodium pyruvate (lane 4). The control condition, to which only water was added, is presented in lane 1. At the 5 mM concentration, dichloroacetic acid causes a 75.5% decrease

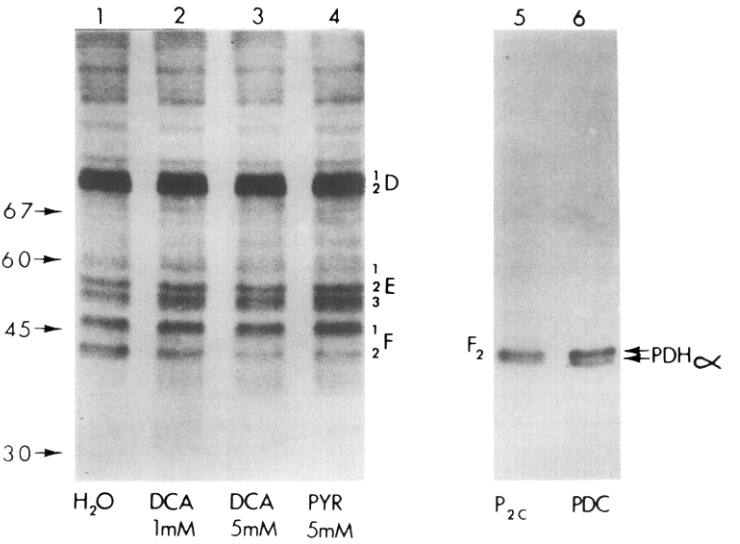

Figure 1: Selective inhibition of band F_2 by dichloroacetate and pyruvate.

In lanes 1-4, brain homogenate protein, at a concentration of 1 mg/ml was reacted with 0.5 mM [^{32}P] ATP for 30 seconds. The reaction was quenched by solubilizing the proteins in SDS. The solubilized material was electrophoresed on polyacrylamide gels and exposed to X-ray film to produce the autoradiogram shown in Figure 1. The material in lane 1 had no additions. Lanes 2 and 3 contain material reacted in the presence of 1 mM and 5 mM dichloroacetate, respectively. In lane 4, pyruvate was added at a concentration of 5 mM. The locations of the major phosphoprotein bands are indicated along the side of the figure. The densitometric results on band F_2 are shown in Table 1.

In lanes 5 and 6, brain mitochondria (lane 5) or purified bovine kidney pyruvate dehydrogenase complex (lane 6) were phosphorylated at 1 mg/ml or 0.1 mg/ml protein concentration respectively. The conditions were identical to those described in Methods except that the EDTA concentration was 8 mM rather than 1 mM. We have found that when EDTA is present in excess of Mg^{2+}, only band F_2 is phosphorylated.

in the densitometric area of band F_2. Sodium pyruvate, at the same concentration, reduces the optical density of the M_r=41,000 band by 85.3%. In contrast, densitometric analysis of bands other than F_2 in lanes 1 through 4 reveals that no other bands vary from control values by more than 15%

The comigration of band F_2 with purified kidney pyruvate dehydrogenase α-subunit is also shown in Figure 1. Protein from a brain mitochondrial fraction (P_{2c}) was phosphorylated under conditions where only band F_2 incorporates phosphate (see Figure legend). The purified pyruvate dehydrogenase complex from bovine kidney was also phosphorylated under these conditions and both

samples were electrophoresed on 10% polyacrylamide gels. Both samples produce doublet bands of M_r=41,000-42,000 on the autoradiogram.

The effect of dichloroacetate on band F_2 is concentration dependent. In Figure 1, the reduction in F_2 phosphorylation by 1 mM dichloroacetate is 50% less than the decrease observed at the 5 mM concentration (quantified densitometrically). In Figure 2, the effects of varying the dichloroacetic acid con-

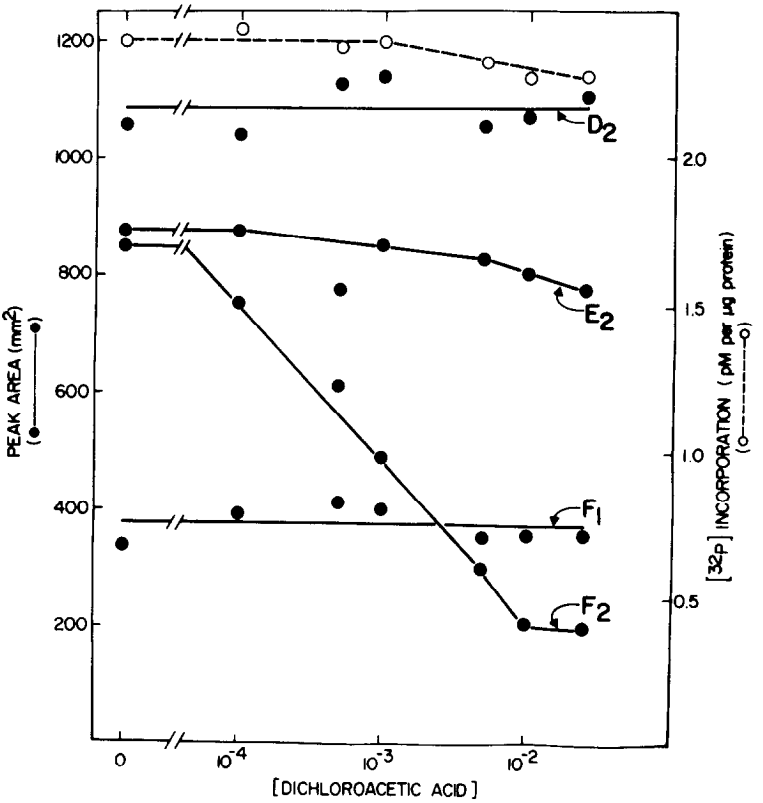

Figure 2: The effects of increasing concentrations of dichloroacetic acid on brain protein phosphorylation.

Brain homogenates at a protein concentration of 2.1 mg/ml were reacted with [32P] ATP for 30 seconds in the presence of several dichloroacetic acid concentrations. Aliquots were removed for polyacrylamide gel electrophoresis and TCA precipitation of protein. In Figure 2 the effects of increasing concentrations of dichloroacetate on the total [32P] incorporation into TCA precipitates and the incorporation of radioactivity into individual phosphoprotein bands (quantified densitometrically) are presented. The solid lines indicate the area of the individual peaks in mm^2. The TCA precipitable [32P] incorporation is indicated by the dashed line. The concentrations of dichloroacetate used were 0, 0.1, 0.5, 1.0, 5.0, 10.0 and 25.0 mM.

centration on the $[^{32}P]$ incorporation into several representative phosphoprotein bands is shown. Between 0.1 and 10 mM dichloroacetate there is a dramatic decrease in the phosphorylation of the 41,000 dalton protein. Since no other bands were consistently affected, it appears that only band F_2 is sensitive to dichloroacetate over this concentration range.

In addition to dichloroacetate and pyruvate, NADH, NAD, acetyl CoA and CoA are known to regulate pyruvate dehydrogenase phosphorylation (12). High levels of NADH and acetyl CoA stimulate phosphorylation, while high concentrations of their reciprocal cofactors, NAD and CoA, inhibit pyruvate dehydrogenase kinase. The effects of these compounds on band F_2 phosphorylation are shown in Table 1. Both acetyl CoA and NADH produce small elevations in band F_2 phosphorylation which appear to be partially additive. When CoA or NAD are added to the in vitro reaction mixture, larger effects on F_2 phosphorylation are

Table 1

Compound	Percent Change from H_2O Control
Acetyl CoA	+ 10.5 %
NADH	+ 23.6
Acetyl CoA + NADH	+ 28.0
CoA	- 12.6
NAD	- 50.0
CoASH + NAD	- 63.1
DCA (1 mM)	- 35.3
DCA (5 mM)	- 73.5
Pyruvate (5 mM)	- 85.3

Regulation of band F_2 phosphorylation by compounds known to modulate pyruvate dehydrogenase kinase activity.

Brain homogenates were reacted with $[^{32}P]$ ATP and the amount of phosphate incorporated into band F_2 was measured densitometrically (see Methods). In the upper portion of the table the effects of CoA-SH, NAD and NADH are given. These compounds were present at a final concentration of 1 mM in the reaction mixture. The lower portion of the table contains the densitometric analysis of the data presented in Figure 1. The final concentrations of these compounds are given in parentheses. The effects of these compounds on band F_2 are expressed as the percent increase or decrease in peak area relative to H_2O controls. NaCl at concentrations up to 50 mM had no effect on band F_2 phosphorylation.

observed. NAD, at a concentration of 1 mM, decreases the densitometric area of band F_2 by 50%. When 1 mM CoA was combined with 1 mM NAD, a further reduction of 13% was observed, which is equal to the inhibition obtained when CoA is added without NAD. The other phosphoproteins were unaltered by these compounds. Therefore, in brain homogenates the phosphorylation of band F_2 is affected by the in vitro concentrations of NAD, NADH, CoA and acetyl CoA.

Discussion

The activity of the mitochondrial enzyme pyruvate dehydrogenase in both neuronal and non-neural tissues is regulated by a cycle of phosphorylation (inactivation) and dephosphorylation (activation) (7,13). The phosphorylated component of the pyruvate dehydrogenase complex is the α-subunit (M_r=41,500), which is involved in the pyruvate decarboxylation step of the reaction sequence. Three phosphates can be incorporated per subunit, although a single phosphomonoester per subunit is sufficient to block enzymatic activity (15). The protein kinase which catalyzes the phosphorylation of the α-subunit is tightly bound to the enzyme complex and inhibited by pyruvate, dichloroacetate, NAD and CoA (10,11,12). NADH and acetyl CoA have been reported to stimulate pyruvate dehydrogenase kinase (12).

The phosphorylation of an M_r=41,000 brain phosphoprotein referred to in prior reports as band F_2, is regulated in a manner identical to that of pyruvate dehydrogenase. We propose, therefore, that band F_2 is the α-subunit of pyruvate dehydrogenase. The evidence for this proposal is as follows: 1) There is a dramatic and selective inhibition of band F_2 phosphorylation by pyruvate, dichloroacetate, NAD and CoA. In addition, NADH and acetyl CoA stimulate $[^{32}P]$ incorporation into this band (Table 1). 2) Recent reports concerning the subcellular localization of phosphorylated brain proteins indicate the enrichment of an M_r=40,000-45,000 phosphoprotein band in mitochondrial fractions (16,17). We have confirmed this mitochondrial localization for band F_2. 3) The α-subunit of purified bovine kidney pyruvate dehydrogenase complex comigrates with band F_2 on sodium dodecyl sulfate polyacrylamide gels (Figure 1). 4) We have recently

found that the decarboxylase activity of pyruvate dehydrogenase is highly correlated with the degree of band F_2 phosphorylation, and sensitive to the treatments reported here (Morgan and Routtenberg, in preparation).

It may be seen in Figure 1 that band F_2 can be resolved into a doublet. This raises the possibility that two or more different phosphorylated proteins may be present in this gel region. However, it should be noted that both bands are inhibited by dichloroacetic acid or pyruvate (Figure 1) and that an 85% reduction of these bands can be obtained (Table 1). Moreover, the purified α-subunit of kidney pyruvate dehydrogenase also migrates as a doublet when phosphorylated. Therefore, it is more probable that both bands in the F_2 region represent the α-subunit rather than different phosphoproteins.

The present hypothesis, then, that band F_2 is pyruvate dehydrogenase should be valuable in defining the role played by this enzyme in regulating functional alterations of neuronal activity in brain.

Acknowledgements

We wish to thank Dr. Lester Reed and Dr. Flora Pettit for the generous supply of pyruvate dehydrogenase complex and the alpha subunit. We are also indebted to them for their constructive suggestions.

This research was supported by N.S.F. BNS79-20630 and N.I.M.H. 25281 to A. R. Gratitude is expressed to Betty Wells for preparation of the manuscript.

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