

Research report

## 2D-PAGE Analysis: adrenergically regulated pineal protein AIP 37/6 is a phosphorylated isoform of cytosolic malate dehydrogenase

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### Abstract

The adrenergic transmitter norepinephrine (NE) dramatically increases the prominence of only two out of the hundreds of [<sup>35</sup>S]methionine-labeled pineal proteins resolved by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). One of these regulated proteins is AIP 37/6 (37 kDa, *pI* ~ 6). The labeling of this protein is increased ~ 100-fold by NE. In the study presented here the identity of AIP 37/6 was investigated. The results of microsequencing, immunochemical analysis of 2D-PAGE blots and size exclusion chromatography indicate that AIP 37/6 is an isoform of cytosolic malate dehydrogenase (cMDH; ~ 36.3 kDa; *pI* ~ 6.5). Associated studies indicate that this isoform is phosphorylated whereas the bulk of cMDH is not. Cotranslational phosphorylation of cMDH is discussed.

**Keywords:** Pineal; Cyclic AMP; Malate dehydrogenase; Phosphorylation; Aspartate/malate shuttle

### 1. Introduction

Many aspects of mammalian pineal function are activated at night by norepinephrine (NE). Release of NE is regulated by a circadian oscillator located in the suprachiasmatic nucleus (SCN) [21]. This oscillator is entrained to the day/night cycle by light acting through retinal → SCN projections [1,17]. The SCN → pineal circuit passes through central and peripheral structures; NE is released into the pineal extracellular space by projections from the superior cervical ganglia [14].

NE has a broad range of effects on second messengers, protein phosphorylation and several proteins in the pineal gland. Most notable are those involved in adrenergic signal transduction and melatonin synthesis. NE increases cyclic AMP through an action involving both  $\alpha_1$ - and  $\beta$ -adrenergic receptors and increases  $[Ca^{2+}]_i$  and inositol phosphate turnover through  $\alpha_1$ -adrenergic mechanisms [14,15]. The increase in cyclic AMP causes rapid phosphorylation of the cyclic AMP response element binding protein (CREB) [25]. Candidate genes for CREB dependent ex-

pression are the immediate early genes Fos-related antigen-2 (*Fra-2*) [2] and the inducible cyclic AMP binding element repressor (ICER) [28], both of which might negatively regulate expression of other genes, including arylalkylamine *N*-acetyltransferase (AA-NAT) [16]. AA-NAT is another candidate for CREB-dependent regulation. It is of central importance in pineal and circadian biology because it exhibits a 100-fold night/day rhythm in activity which drives the nocturnal rhythm in melatonin synthesis seen in vertebrates [18]. The interplay of positive influences of CREB and negative influences of other factors might contribute to the rhythm in AA-NAT activity.

Cyclic AMP also stimulates phosphorylation of phosphodiesterase (MEKA) [26], a phototransduction protein of unknown function in the pineal gland, influences the biosynthesis of biopeterin [13], a cofactor required for the synthesis of melatonin, and enhances expression of thyroxine 5'-deiodinase activity [22]. The importance of the latter effect in the pineal gland is unknown.

The downstream effects of  $\alpha_1$ -adrenergic-regulated second messengers on protein synthesis in part reflect potentiation of  $\beta$ -adrenergic stimulation of adenylate cyclase [14]. This is mediated by  $Ca^{2+}$ -, phospholipid-dependent protein kinase [15]. A clear example of an effect of

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$\alpha_1$ -adrenergic activation which is not cyclic AMP-dependent is stimulation of *c-fos* expression [6]. NE also acts, apparently through a  $\beta$ -adrenergic receptor mechanism, to increase the activity of the last enzyme in melatonin synthesis, hydroxyindole-*O*-methyltransferase [29].

In view of the broad and dramatic effects NE has on pineal metabolism, it is remarkable that two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the hundreds of [ $^{35}$ S]methionine-labeled pineal proteins has detected dramatic changes in the synthesis of only two proteins [30]. Neither protein has been identified; one was termed AIP 37/6, for adrenergically-induced 37 kDa protein with  $pI \sim 6$  [30]. The abundance of [ $^{35}$ S]methionine-labeled AIP 37/6 increases  $\sim 100$ -fold through an adrenergic  $\rightarrow$  cyclic AMP post-transcriptional mechanism, without significantly increasing the total amount of AIP 37/6 [30]. In the studies presented here AIP 37/6 was determined to be a phosphorylated isoform of cytosolic malate dehydrogenase (cMDH).

## 2. Materials and methods

### 2.1. Tissue

Frozen rat pineal glands (Sprague-Dawley; day killed; mixed age and sex; referred to as day pineal glands) were obtained from Zivic Miller Laboratories (Allison Park, PA). Pineal glands used for organ culture experiments were obtained from 250 g female rats (Sprague-Dawley, Taconic Farms, Germantown, MD).

### 2.2. Organ culture

Glands were incubated in organ culture following a published procedure [24] and were adrenergically stimulated with a mixture of isoproterenol (0.1  $\mu$ M) and phenylephrine (1  $\mu$ M). Proteins were labeled with [ $^{35}$ S]methionine by incubation in methionine-free medium for the 48 to 50 h period and in medium containing [ $^{35}$ S]methionine (200  $\mu$ Ci/ml, 0.3–0.6  $\mu$ M) with or without adrenergic stimulation for the 50–53 h period. Cultured glands were frozen on dry ice immediately following treatment. Proteins were labeled with  $^{32}$ P<sub>i</sub> by incubation in P<sub>i</sub>-free BGJ<sub>b</sub> medium for 1 h followed by a 6 h incubation in the same medium containing trisodium  $^{32}$ P<sub>i</sub> (2 mCi/ml) with or without adrenergic drugs.

### 2.3. Tissue preparation

Glands were homogenized in 10 volumes of 100 mM sodium citrate, pH 6.5, containing 1.0 mM dithiothreitol (DTT), leupeptin (2  $\mu$ M) and phenylmethylsulfonyl fluoride (0.5 mM). Homogenates were centrifuged (100,000  $\times$  g, 1 h, 4°C) and the supernatants were retained for further analysis unless otherwise indicated. Adrenergic stimulation was verified by analysis of AA-NAT activity [24].

Supernatants from control and adrenergically-stimulated [ $^{35}$ S]methionine-labeled glands were concentrated (10 k Microsep centrifugal concentrator; Filtron Technology Corp., Northborough, MA) if they were to be used for 2D-PAGE without size exclusion chromatography. Aliquots were removed for determination of protein [4], incorporation of radiolabel into protein [30] and AA-NAT activity [24].

To prepare samples for 2D-PAGE and microsequencing, gel exclusion chromatography was used to obtain an enriched preparation of AIP 37/6. Day pineal and adrenergically-stimulated pineal supernatants (100,000  $\times$  g, 1 h, 4°C) were mixed (4:1); the latter provided a source of AA-NAT activity. A 2.0 ml sample was chromatographed on a preparative TSK-G3000SW (21.5 mm  $\times$  60 cm) column (Thompson Instrument Co., Springfield, VA); the mobile phase was 100 mM sodium citrate, pH 6.5, containing 1.0 mM DTT (1.5 ml/min; 3 ml/fraction, 4°C). Fractions containing  $\sim 37$  kDa proteins were selected based on retention of molecular weight protein size standards (data not shown). The proteins in these fractions were size-selected (Macrosep 100 k centrifugal concentrator). The filtrate was desalted and concentrated (Macrosep 10 k centrifugal concentrator). Retenates from two chromatographic runs were pooled (1.3 mg protein), lyophilized and immediately solubilized in Buffer I (0.3% SDS, 200 mM DTT, 28 mM Tris-HCl and 22 mM Tris-base). These samples were then boiled for 5 min and cooled on wet ice, and four volumes of Buffer III (9.9 M urea, 4.4% Nonidet P-40, 2.2% ampholytes, pH 4–8, and 100 mM DTT) were added (final protein concentration  $\sim 3.2$   $\mu$ g/ $\mu$ l). To locate AIP 37/6 in the 2D-PAGE pattern it was necessary to generate an internal reference pattern of [ $^{35}$ S]methionine-labeled proteins. This was done by adding a sample of [ $^{35}$ S]methionine-labeled protein ( $\sim 150$   $\mu$ g,  $\sim 3.5 \times 10^6$  DPM, see above) supernatant from adrenergically-induced pineal glands to the size selected sample containing 37 kDa proteins (650–800  $\mu$ g). The sample containing the labeled proteins was resolved by preparative 2D-PAGE as described below.

Analytical size exclusion high performance liquid chromatography (HPLC) was conducted using a TSK-G3000SW (7.5 mm  $\times$  60 cm) column, as described in the legend to Fig. 4. cMDH immunoreactivity was monitored by dot blot analysis and cMDH enzyme activity was determined by a colorimetric assay as described below. The column was calibrated using albumen (67 kDa, fraction 32) and chymotrypsinogen (25 kDa, fraction 38). MDH activity peaked in fraction 36, which is predicted to be enriched with 35 kDa proteins.

### 2.4. 1D-PAGE

Procedures described in detail elsewhere were followed [19].

## 2.5. 2D-PAGE

The procedures used for analytical and preparative 2D-PAGE were based on a published method [23] and are described in the Millipore Investigator 2-D Electrophoresis Manual (MilliGen/Biosearch Division of Millipore, Burlington, MA). The equipment and reagents used were from Millipore unless otherwise indicated.

### 2.5.1. First dimension

Analytical isoelectric focusing (IEF) gels contained 9.5 M urea, 2.0% (v/v) Nonidet P-40, 4.1% Duracryl HTL (30:0.8 acrylamide: bis-acrylamide), 5 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and 2.3% ampholytes (pH 4 to 8). Analytical gels were prefocused for approximately 2.5 h. Analytical samples were focused for 18,000 Vh. Preparative IEF gels contained 8 M urea, 2.0% poly-B-D-glucopyranoside, 5.5% ampholytes (pH 4 to 8), 10 mM CHAPS and 4.4% Duracryl HTS (30:0.8 acrylamide: bis-acrylamide) solution. After prefocusing the gels for 5 h, the samples were focused for 17,500 Vh. The useful resolving range of the electrofocusing gel was pH 4 to 8 as determined by 2D-PAGE standards (Bio-Rad Laboratories, Richmond, CA).

### 2.5.2. Second dimension

Tube gels were extruded and equilibrated in 0.3M Tris base, 0.075 M Tris-HCl, 3.0% SDS, 50 mM DTT and 0.01% Bromophenol blue, pH 8.6–9.3 (2 min) and preparative tube gels were equilibrated in 0.3 M Tris-HCl, 8 mM Tris base, 3.0% SDS, 50 mM DTT, 0.01% Bromophenol blue, pH 6.6–7.0 (2 × 15 min). The gels were positioned on a 12.5% Duracryl HTS (30:0.8 acrylamide:bis-acrylamide) sizing gel (25 cm × 25 cm) with (preparative) or without (analytical) a 3 cm stacking gel. The IEF gel was secured with 1% IEF Agarose (Pharmacia LKB Biotechnology, Piscataway, NJ). The running buffer was 25 mM Tris-base, 192 mM glycine, and 0.1% SDS. The sizing gel was run at 1800 V for ~ 18 h at 15°C. The size standards were Rainbow markers ( $M_r$  14,300–200,000; Amersham, Arlington Heights, IL).

### 2.5.3. Transblotting

Proteins were electroblotted (~ 18 h in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid buffer, pH 11, containing 10% methanol) onto ProBlott™ membrane (Applied Biosystems, Foster City, CA) for microsequencing or onto Immobilon P (Millipore Corp., Bedford, MA) for immunochemical detection. Blots were stained in 0.1% Amido black in 40% methanol/1% acetic acid and destained in water.

## 2.6. Localization of AIP 37/6 in 2D-PAGE pattern

AIP 37/6 was located in the autoradiographic patterns of [<sup>35</sup>S]methionine-labeled proteins based on evidence of

adrenergically-stimulated labeling of a ~ 37 kDa,  $pI$  ~ 6 protein in the published pattern of labeled pineal proteins (Fig. 1) [23]. It was also possible to locate AIP 37/6 in the Amido black stained pattern of 37 kDa enriched proteins by reference to the standard autoradiographic pattern produced by the added [<sup>35</sup>S]methionine-labeled proteins. The designations 37/7 and 37/8 were used to identify abundant neighboring proteins (Fig. 1, Table 1).

## 2.7. Microsequencing

Sections of Amido black stained membrane were excised, washed and incubated with trypsin (modified sequencing grade, Boehringer Mannheim, Indianapolis, IN) [11]. Digests were chromatographed on a narrowbore (2.1 × 250 mm) Vydac 218TP52 column and guard column at 35°C on a System Gold HPLC (Beckman Instrument Co., Fullerton, CA) equipped with a Model 507 autosampler, Model 126 programmable solvent module and Model 168 diode array detector. Fractions (125 μl) containing tryptic peptides were applied in 30 μl aliquots to a Biobrene treated glass fiber filter (Applied Biosystems, Foster City, CA) and dried. These were subjected to automated Edman degradation in a blot cartridge on a Model 477A pulsed-liquid protein sequencer equipped with a Model 120A PTH analyzer using the manufacturer's methods and cycles; data were collected and analyzed on a Model 610A data analysis system (Applied Biosystems). Amino acid sequences were searched in the Genetics Computer Group Swiss-Prot database (University of Wisconsin Genetics Computer Group, Madison, WI) [7].

## 2.8. Immunochemical techniques

### 2.8.1. Antisera

A dialyzed γ-globulin fraction of a goat anti-mouse cMDH (anti-cMDH) prepared by NH<sub>4</sub>SO<sub>4</sub> fractionation was provided by Dr. M. Felder, Department of Biology, University of South Carolina, Columbia, SC [10]. This was used for immunodetection and immunoprecipitation. Where indicated, anti-cMDH was immunopurified by absorbing antibodies to sections of blots containing cMDH<sub>37/8</sub> (see 3.2) and eluting with 0.1 N HAc containing 0.1% bovine serum albumin [27]. Rabbit anti-mouse cMDH<sub>273–283</sub> serum was raised (HL 1559; anti-cMDH<sub>273–283</sub>; Hazelton Laboratories, Vienna, VA) against mouse cMDH<sub>273–283</sub> (DGNSYGVPPDDL; University of Notre Dame Bioscience Core Facility, Notre Dame, IN). Rabbit anti-porcine mitochondrial malate dehydrogenase (mMDH) [8] was provided by Dr. T.H. Oh, Department of Anatomy, University of Maryland Medical School, Baltimore, MD.

### 2.8.2. Immunoprecipitation

A 200 μl sample of supernatant containing radiolabeled proteins was mixed with 200 μl of crude anti-cMDH serum and 1.6 ml of phosphate-buffered saline (2.7 mM

Table 1  
Results of microsequencing

Protein	Sequence	Yield <sup>a</sup>	Identification from Database <sup>b</sup>	$M_r$	Amino acid no.
AIP 37/6	XXFITTVQQ	7.2	cMDH, mouse	36.3	220–228
	XEFITTVQQ	2.5	cMDH, mouse	36.3	220–228
37/7	XEFITTVQQR	10.6	cMDH, mouse	36.3	220–229
37/8	VIVVGNPANTNXLTASK	32.5	cMDH, mouse	36.3	127–143

A fraction of rat pineal proteins enriched in AIP 37/6 was analyzed by 2D-PAGE. Sections of the blot containing AIP 37/6 and other neighboring 37 kDa proteins were excised, subjected to tryptic digestion, and microsequenced as described in Section 2.

<sup>a</sup> Initial yield based on least square analysis of lag corrected yield (pmol) at each cycle versus cycle number.

<sup>b</sup> Protein homology was determined by searching the Swiss-Prot database using the FASTA program contained in the Genetics Computer Group Sequence Analysis Package [7].

Na<sub>2</sub>HPO<sub>4</sub>, 1.6 mM KH<sub>2</sub>PO<sub>4</sub>, 154 mM NaCl, pH 7.2) containing 1 mM NaF and the tube was rotated (5 rpm; 2 h, 23°C + 18 h, 4°C). The precipitated antibody/antigen complex (15 min, 14,000 × *g*) was washed and protein samples (2 μg/μl) containing cMDH from ~0.5 (Fig. 2, Fig. 3) or ~2.5 pineal glands (Fig. 5, Fig. 6) were analyzed by 2D-PAGE. No precipitate formed when normal goat serum was used.

### 2.8.3. Immunodetection

Membranes were incubated (18 h) with immunopurified goat anti-mouse-cMDH (1:100 or 1:200) or anti-cMDH<sub>273–283</sub> (1:1000 or 1:6000). In the case of anti-cMDH, the immunocomplex was visualized autoradiographically (Fig. 3, Fig. 4, Fig. 6) using rabbit anti-goat IgG conjugated to alkaline phosphatase (H + L; 40 min; 0.5 μg/ml; Kirkegaard and Perry Laboratories, Gaithersburg, MD) followed by [<sup>125</sup>I]-rProtein A (9 μCi/μg; 200,000 dpm/ml; DuPont Co., Wilmington, DE). In the case of anti-cMDH<sub>273–283</sub>, the immunocomplex was visualized chromatogenically (1:1000, 18 h) in Fig. 3 using goat anti-rabbit IgG conjugated to alkaline phosphatase (0.5 μg/ml; Kirkegaard and Perry Laboratories) or detected by enhanced chemiluminescence (1:6000, 18 h) in Fig. 6 following incubation with goat anti-rabbit IgG conjugated to horseradish peroxidase (0.025 μg/ml; Kirkegaard and Perry Laboratories) using ECL™ (Amersham Corp., Arlington Heights, IL). Signals were not detected if preimmune serum was used in place of the antisera identified above.

### 2.8.4. Dot blot analysis

To determine which HPLC fractions contained cMDH, 50 μl samples were dot blotted onto Immobilon-P. Immunochemical detection was as described above with anti-cMDH (1:10,000) or anti-cMDH<sub>273–283</sub> (1:2000). The immunocomplex was visualized by enhanced chemiluminescence following exposure to goat anti-rabbit or rabbit anti-goat IgG conjugated to horseradish peroxidase (0.025 μg/ml).

### 2.9. MDH assay

MDH activity was monitored in samples of fractions by measuring the difference in the rate at which MTT (3-

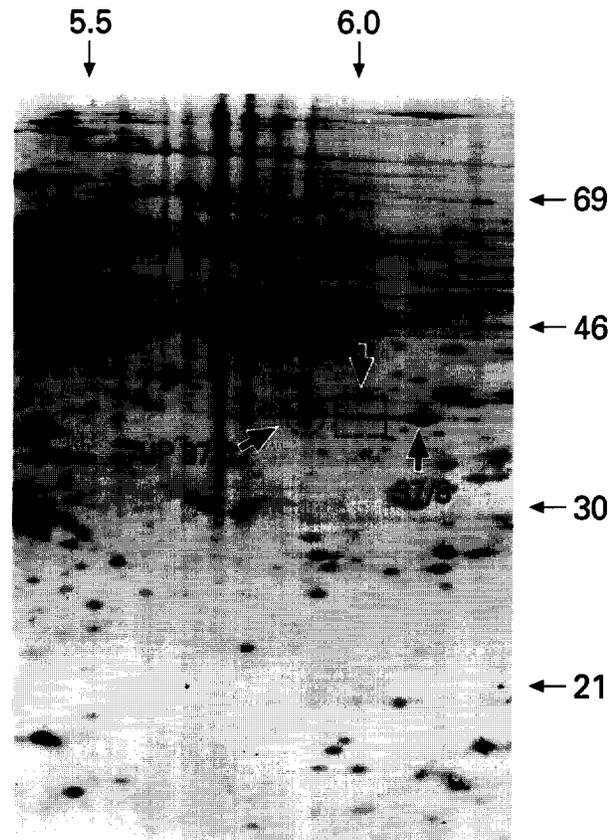


Fig. 1. Typical autoradiographic pattern of 2D-PAGE resolved [<sup>35</sup>S]methionine-labeled proteins prepared from adrenergically-stimulated rat pineal glands. Pineal glands were cultured with [<sup>35</sup>S]methionine in the presence of isoproterenol and phenylephrine. Samples (~500 μg of 100,000 × *g* supernatant protein) were resolved by preparative 2D-PAGE as described in Section 2. A comparison of control (not shown) and induced protein patterns was used to identify AIP 37/6 (broken circle) for microsequencing. Proteins 37/7 (broken box) and 37/8 were also subjected to microsequence analysis (see Table 1). For further details see Section 2.

[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is reduced by enzymatically generated NADH in the presence and absence of L-malate [5]. A 50  $\mu$ l sample of a fraction was added to wells of a 96-well microtiter plate containing 50  $\mu$ l 50 mM HEPES, pH 7.5,  $\pm$ 10 mM L-malate. Reactions were initiated by adding 100  $\mu$ l of 25 mM HEPES, pH 7.5, 5 mM NAD<sup>+</sup>, 5 mM MTT, and 5 mM phenazine methosulfate (PMS) to the wells. Following incubation (20 min, 25°C), the absorbance at 595 nm was determined using a Bio-Rad model 2550 EIA reader (Bio-Rad Laboratories, Richmond, CA). The difference between the absorbance plus L-malate and minus L-malate was used to calculate MDH activity ( $\epsilon_{595} = 5.8 \text{ mM}^{-1} \text{ min}^{-1}$ ). A standard curve was generated with known amounts of NADH.

### 3. Results

#### 3.1. Microsequencing indicates AIP 37/6 is a form of cMDH

A fraction of pineal protein enriched with AIP 37/6 was prepared and proteins were resolved by 2D-PAGE. AIP 37/6 and other  $\sim$ 37 kDa proteins of interest were identified (Fig. 1). Microsequencing of tryptic peptides

revealed that AIP 37/6 and the more abundant proteins identified as 37/7 and 37/8 share sequence with cMDH (Table 1), indicating all are cMDH isoforms. This is consistent with the predicted mass ( $\sim$ 36.3 kDa) and pI ( $\sim$ 6.5) of mammalian cMDH based on the deduced amino acid sequence, with the reported apparent mass of cMDH, and with evidence that cMDH exists in several isoforms [7,10,12].

#### 3.2. Immunochemical confirmation that AIP 37/6 is a cMDH isoform

The indication that AIP 37/6 is cMDH was examined immunochemically using an anti-holoprotein serum (goat anti-cMDH). Proteins were immunoprecipitated using crude anti-cMDH and resolved by 2D-PAGE. Autoradiographic analysis revealed adrenergically-induced differences in [<sup>35</sup>S]methionine-labeling of AIP 37/6 in the immunoprecipitate (Fig. 2).

AIP 37/6 and proteins 37/7 and 37/8 were immunopositive (Fig. 2). The intensity of the immunostaining of AIP 37/6 in control and adrenergically-stimulated samples was not different (Fig. 2), consistent with results obtained using silver stain which indicated that NE increases [<sup>35</sup>S]methionine-labeling of AIP 37/6 without increasing total protein [30].

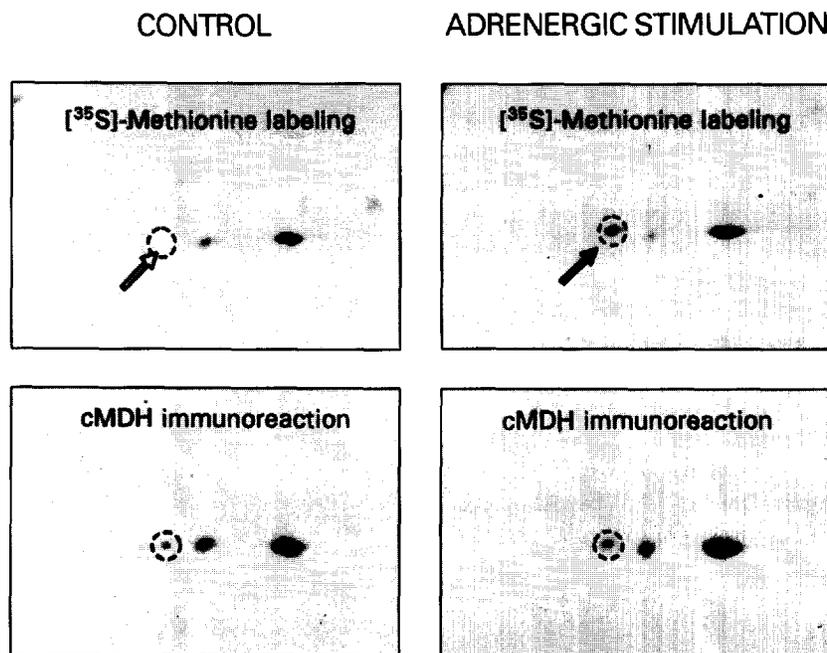


Fig. 2. Autoradiographs and anti-cMDH immunopatterns of 2D-PAGE patterns of [<sup>35</sup>S]methionine-labeled anti-cMDH immunoprecipitates of cytosolic proteins prepared from control and adrenergically-stimulated rat pineal glands. [<sup>35</sup>S]Methionine-labeled proteins in control and stimulated rat pineal supernatants ( $10,000 \times g$ ) were immunoprecipitated with anti-cMDH serum. The immunocomplex was solubilized in sample buffer. Samples containing cMDH from  $\sim$ 0.5 pineal gland were resolved by analytical 2D-PAGE and transblotted onto Immobilon P. Autoradiography (7 day exposure) revealed that [<sup>35</sup>S]methionine-labeled AIP 37/6 was not present in the control (top left) but was present following adrenergic stimulation (top right). Following a 2-month period the radioactive signals were not detectable during a 24 h exposure. At this time cMDH was immunodetected using immunopurified anti-cMDH (1:100) serum, exposed to [<sup>125</sup>I]-rProtein A and autoradiographs (24 h) were prepared. Immunochemical analysis revealed no difference between control (bottom left) and stimulated cMDH protein patterns (bottom right). Position of cMDH<sub>37/6</sub> is indicated by broken circle. For further details see Section 2.

These studies were extended using an anti-peptide serum raised against cMDH<sub>273–283</sub> (anti-cMDH<sub>273–283</sub>). This generated a single ~37 kDa band of protein on a 1-D Western blot (data not shown) of total pineal protein, indicating that the antiserum was highly specific. This was then used for 2D-PAGE analysis of immunoprecipitated cMDH. Anti-cMDH<sub>273–283</sub> generated the same immunopattern obtained with anti-cMDH (Fig. 3), confirming that AIP 37/6 and the other proteins in the immunopositive cluster are cMDH isoforms. Accordingly, AIP 37/6, 37/7 and 37/8 are referred to below as cMDH<sub>37/6</sub>, cMDH<sub>37/7</sub> and cMDH<sub>37/8</sub>. In addition to these three isoforms, more acidic isoforms were detected with larger amounts of immunoprecipitated cMDH (Fig. 5).

Anti-cMDH<sub>273–283</sub> and anti-cMDH sera were also used to analyze fractions of an analytical size exclusion chromatographic separation (Fig. 4), in which MDH activity was monitored to confirm that immunoreactive cMDH coeluted with MDH enzyme activity. Each antiserum generated a pattern characterized by a peak in immunostaining coinciding with a peak in MDH activity (fraction 36).

### 3.3. cMDH<sub>37/6</sub> is a phosphoprotein

Differences in the isoelectric point of each isoform might in part reflect independent differences in post-trans-

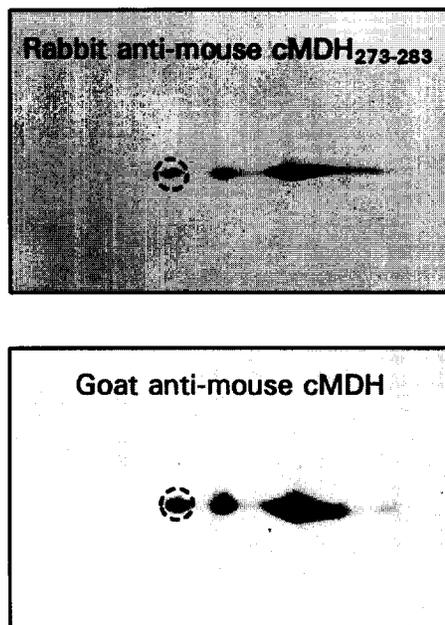


Fig. 3. Anti-cMDH<sub>273–283</sub> and anti-cMDH identify the same proteins in anti-cMDH immunoprecipitates of pineal proteins. [<sup>35</sup>S]Methionine-labeled pineal 100,000 × g supernatant was prepared and immunoprecipitated with anti-cMDH serum. Samples of the protein-antibody complex equivalent to ~0.5 pineal gland were resolved by analytical 2-D PAGE. The proteins were transblotted onto Immobilon P and processed for autoradiography followed by immunochemical detection. Anti-cMDH<sub>273–283</sub> (top) and immunopurified anti-cMDH serum (bottom) produced the same pattern. Position of cMDH<sub>37/6</sub> is indicated by broken circle. For further details see Section 2.

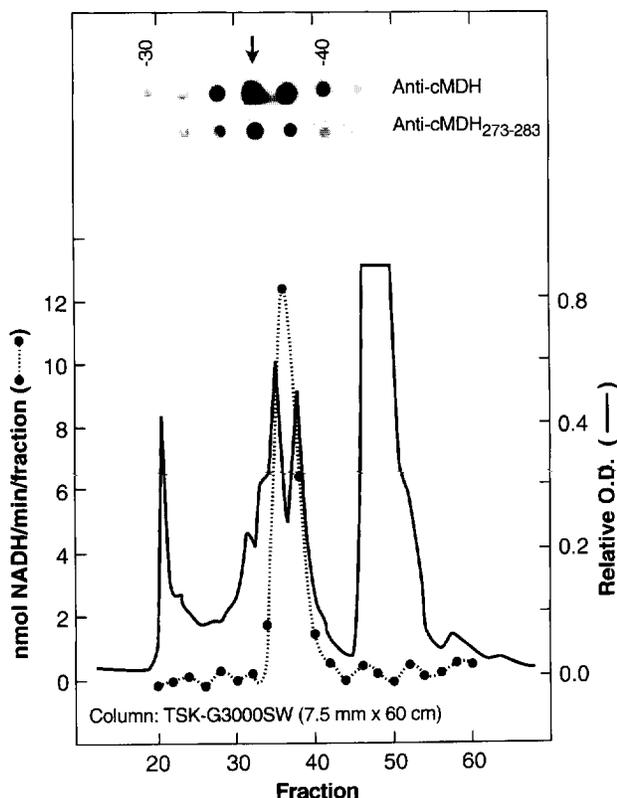


Fig. 4. MDH activity and cMDH coelute in cytosolic pineal preparations. Night rat pineal supernatant (1.3 mg, 100,000 × g, 1 h, 4°C) was chromatographed on an analytical TSK-G3000SW (7.5 mm × 60 cm) HPLC column equilibrated with 100 mM sodium citrate, pH 6.5. The protein profile (solid line) was determined by continuously measuring eluate absorbance at 280 nm. MDH activity (broken line) was determined as described in Section 2. The peak of enzyme activity in fraction 36 corresponds to peak immunoreactivity for each of the anti-cMDH antibodies tested (arrow); the cMDH antisera did not react with mMDH. MDH activity did not correlate with mMDH which was present in trace amounts in fractions 28–38, as indicated using an anti-mMDH serum [8]; essentially all pineal mMDH was in the 100,000 × g pellet (data not shown). Addition of 1 mM DTT to the column and protein sample buffer had no effect on the position of the peak in MDH activity (data not shown).

lational modifications of cMDH, including phosphorylation. To determine if cMDH<sub>37/6</sub> was phosphorylated, cMDH isoforms from <sup>32</sup>P- or [<sup>35</sup>S]methionine-labeled glands were immunoprecipitated, resolved by 2D-PAGE, and electrotransferred to membrane. cMDH<sub>37/6</sub> was identified by the adrenergically-induced increase in [<sup>35</sup>S]methionine-labeled protein within the cMDH immunopattern. cMDH<sub>37/6</sub> was strongly labeled by <sup>32</sup>P, whereas the major cMDH isoforms, cMDH<sub>37/7</sub> and cMDH<sub>37/8</sub>, were not (Fig. 5). In addition, it was possible to detect faint phosphorylation of a more acidic cMDH isoform, which is not apparent in Fig. 5.

Previous studies indicated that the adrenergically-induced appearance of cMDH<sub>37/6</sub> did not reflect conversion from a prelabeled precursor cMDH<sub>37/6</sub> [30] but clearly required ongoing protein synthesis. We determined whether phosphorylation of cMDH also requires protein synthesis.

This was done by treating glands with cycloheximide (Fig. 6). This treatment blocked the adrenergically-induced increase in  $^{32}\text{P}$ -labeling of cMDH but did not generally inhibit  $^{32}\text{P}$ -labeling of proteins resolved by 1-PAGE (data not presented). The absence of global inhibition of phos-

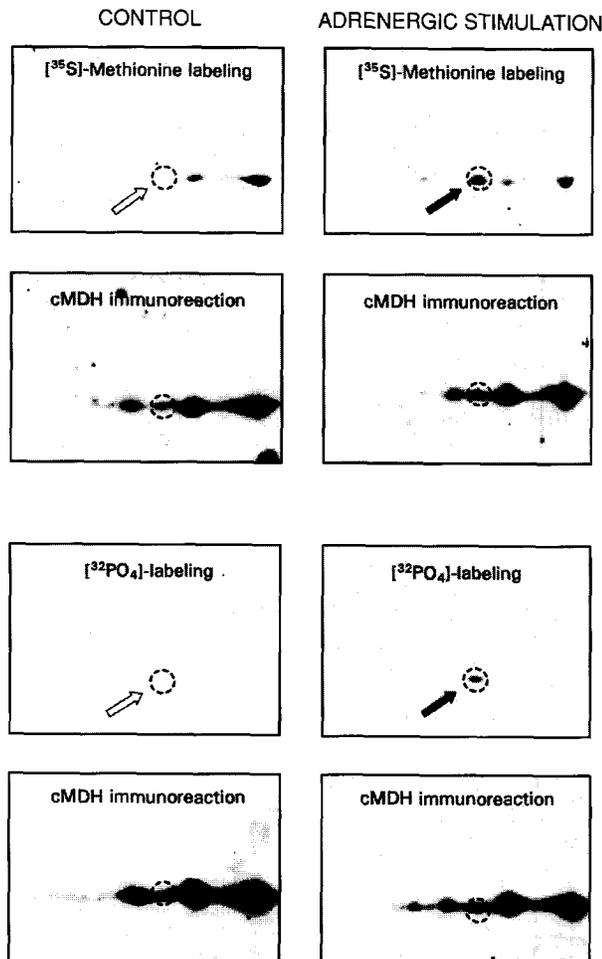


Fig. 5. 2D-PAGE immunoblot and autoradiographic patterns of [ $^{35}\text{S}$ ]methionine- and  $^{32}\text{P}$ -labeled control and adrenergically-stimulated rat pineal proteins immunoprecipitated with anti-cMDH serum. Control and adrenergically stimulated rat pineals were incubated with [ $^{35}\text{S}$ ]methionine or trisodium  $^{32}\text{P}_i$  and immunoprecipitated with anti-cMDH serum. Samples containing cMDH protein from  $\sim 2.5$  pineal glands were analyzed by preparative 2D-PAGE and electroblotted as described in Section 2. Following autoradiography ([ $^{35}\text{S}$ ]methionine, 2.5 days;  $^{32}\text{P}$ , 8 days) blots were stored until radioactivity signals were undetectable during a 2 day autoradiographic exposure. Radioimmunochemical analysis was then performed with immunopurified anti-cMDH serum (1:200) and detected by autoradiography (18 h) as described in Section 2. Upper half: analysis of [ $^{35}\text{S}$ ]methionine-labeled cMDH isoforms from control and adrenergically-stimulated rat pineal glands. An adrenergically-induced increase in [ $^{35}\text{S}$ ]methionine-labeling of cMDH $_{37/6}$  is evident. The immunostained patterns are not different. Lower half: analysis of  $^{32}\text{P}$ -labeled cMDH isoforms in control and adrenergically-stimulated rat pineal glands. An adrenergically-induced increase in  $^{32}\text{P}$ -labeling of cMDH $_{37/6}$  is evident. The immunostained patterns are not different. Position of cMDH $_{37/6}$  is indicated by broken circle. For further details see Section 2.

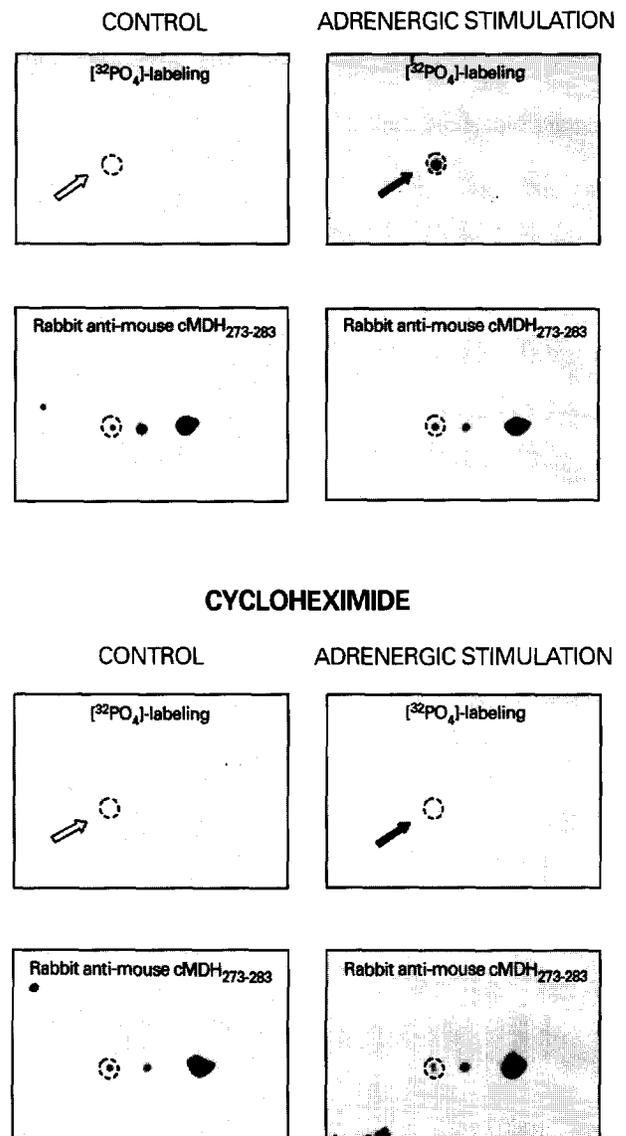


Fig. 6. Cycloheximide blocks appearance of the phosphorylated cMDH isoform cMDH $_{37/6}$ . Rat pineal glands were treated with (lower panel) or without (upper panel) cycloheximide (30  $\mu\text{M}$ ) for 1 h prior to adrenergic stimulation and addition of trisodium  $^{32}\text{P}_i$ . cMDH was immunoprecipitated and samples of the immunoprecipitate containing cMDH protein from  $\sim 2.0$  glands were analyzed by analytical 2D-PAGE and electroblotted as described in Section 2. Following autoradiography (8 day exposure), immunochemical analysis was performed with anti-cMDH $_{273-283}$  serum (1:6000) and detected by enhanced chemiluminescence (2 min exposure). Upper half: analysis of  $^{32}\text{P}$ -labeled cMDH isoforms from control and adrenergically stimulated rat pineal glands. An adrenergically-induced increase in  $^{32}\text{P}$ -labeling of cMDH $_{37/6}$  is evident. The cMDH immunopatterns are not different. Lower half: analysis of  $^{32}\text{P}$ -labeled cMDH isoforms from control and adrenergically stimulated rat pineal glands cultured in the presence of cycloheximide. An adrenergically induced increase in  $^{32}\text{P}$ -labeling of cMDH $_{37/6}$  is not evident. The cMDH immunopatterns are not different. Position of cMDH $_{37/6}$  is indicated by broken circle. For further details see Section 2.

phorylation by cycloheximide suggests that inhibition of phosphorylation of cMDH does not reflect nonspecific inhibition of protein phosphorylation.

#### 4. Discussion

These studies have identified pineal AIP 37/6 as a phosphorylated isoform of cMDH (cMDH<sub>37/6</sub>). This raises two interesting issues. One is the molecular basis of the selective regulation of cMDH<sub>37/6</sub> and the second is the functional significance of it. Although it is not possible to settle these issues without more investigation, these observations merit brief comment.

It is possible to construct a working hypothesis to explain the unusual features of the regulation of cMDH<sub>37/6</sub>. This hypothesis is that the appearance of cMDH 37/6 is due to cyclic AMP-dependent phosphorylation of a small fraction of newly synthesized cMDH. The involvement of cyclic AMP is supported by the observations that NE acts on cMDH through cyclic AMP [30] and that cMDH has a single protein kinase A consensus phosphorylation sequence at cMDH<sub>237–240</sub> (RKLS) [7]. In addition, the catalytic subunit of protein kinase A can phosphorylate peptide cMDH<sub>229–242</sub> (data not shown). Other evidence is that crystallographic studies indicate a charged ion is present in cMDH close to this phosphorylation site [3]. Although this ion was initially identified as sulfate from the available crystallographic evidence, it is equally possible it is phosphate.

The requirement for protein synthesis in the hypothesis is indicated by the findings that NE-induced appearance of <sup>32</sup>P- or [<sup>35</sup>S]methionine-labeled cMDH<sub>37/6</sub> requires protein synthesis (Fig. 6) [30]. The stable nature of the phosphorylation is indicated by the finding that cMDH<sub>37/6</sub> is detectable after long periods without adrenergic stimulation [30]. The indication that only a small fraction of cMDH is phosphorylated is evidenced from <sup>32</sup>P-labeling and immunostaining studies (Fig. 6).

There are several immediate questions raised by this hypothesis, including why the phosphorylation appears to require protein synthesis and why phosphorylation appears to be stable. One scenario which fits with the observations is that phosphorylation only occurs when the phosphorylation site is briefly available, during synthesis. After synthesis, the site might be inaccessible due to folding which would prevent dephosphorylation or phosphorylation. The reason only a small fraction of newly formed cMDH is phosphorylated might be that the opportunity for phosphorylation is limited temporally to the brief period between synthesis of the phosphorylation site and internalization, and is limited kinetically by the availability of the kinase.

An alternative explanation of the mechanism underlying the effects of NE on cMDH<sub>37/6</sub> should also be considered, i.e. that this reflects the induction of an enzyme involved in post-translational modification. This seems unlikely because protein kinase A is abundant.

The functional significance of the adrenergically-stimulated appearance of cMDH<sub>37/6</sub> is not clear. Phosphorylation could alter  $V_{max}$ ,  $K_m$ , or substrate specificity. This could explain the cyclic AMP-induced 50% increase in rat

myotube MDH activity seen following 8 days of treatment [20]. However, we feel it is unlikely that phosphorylation would alter total cMDH activity in the pineal gland because cMDH<sub>37/6</sub> represents a minor fraction of total cMDH protein present. This suspicion is supported by the results of preliminary kinetic analysis of pineal cMDH ( $V_{max}$  and  $K_m$ ) following 6 h of adrenergic stimulation, which failed to reveal any differences (unpublished results). Another hypothetical possibility is that phosphorylation alters association of cMDH<sub>37/6</sub> with other proteins. Such protein/protein association of the mitochondrial form of malate dehydrogenase is thought to enhance the efficiency of substrate/product transfer [9]. We investigated this and were unsuccessful in attempts to obtain evidence of association of cMDH with cytosolic proteins using size exclusion chromatography analysis and non-denaturing gel electrophoresis (unpublished data).

Finally, it is possible that phosphorylation promotes an action of cMDH which enhances the function of this enzyme in the aspartate/malate shuttle. This shuttle effectively transfers AcCoA equivalents from the mitochondria, where it is formed, to the cytosol. The enhanced transfer of AcCoA equivalents to the cytosol would be an advantage to the pinealocyte because of the importance AcCoA plays in melatonin synthesis as a cofactor for AA-NAT. Perhaps phosphorylation enhances docking of cMDH<sub>37/6</sub> at a critical location on the surface of the mitochondria or otherwise enhances the aspartate/malate shuttle, and thereby meets higher AcCoA demands of all cyclic AMP-activated cells. This interesting possibility warrants further detailed investigation.

The effects of adrenergic or cyclic AMP stimulation of the pineal gland on the 2D-PAGE pattern of <sup>35</sup>S-labeled cMDH are striking. It seems likely that similar effects occur in other tissues, and it is hoped that the findings presented here may prove to be useful in the interpretation of similar studies using other tissues.

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