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A Transaldolase

An Enzyme Implicated in Crab Steroidogenesis

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In arthropods, development is controlled by cholesterol-derived steroid hormones: the ecdysteroids. In vertebrates and insects, steroidogenesis is positively regulated and this is mediated by cAMP. In crustaceans, ecdysteroid biosynthesis by steroidogenic organs (Y-organs) is negatively regulated by a neuropeptide, the Molt Inhibiting Hormone (MIH). This neuropeptide-induced inhibition occurs via cyclic nucleotides and depends on protein synthesis. In the present work, we provide evidence that a major 36.2-kDa cytosolic protein (P36; pI: 6.8) from crab Y-organs is positively correlated with steroidogenic activity. On the basis of its amino acid sequence, P36 could be related to transaldolase, an enzyme of the pentose phosphate pathway which generates NADPH. In Y-organs, the enzymatic activity of *Carcinus* transaldolase increases with steroidogenic activity, and MIH treatment decreases both synthesis and activity of transaldolase. Various transaldolases have been characterized in very distantly related groups, namely bacteria, yeasts, and humans. These enzymes are highly conserved and present strong structural homologies, interestingly the crab transaldolase is closest to that enzyme characterized in human cells.

Key Words: Steroidogenesis; transaldolase; crustacea; neuropeptide; 2D electrophoresis.

Introduction

In arthropods (crustaceans and insects), development and growth occur through consecutive molts during which the rigid cuticule is removed and a new one generated. This complex process is under the control of steroid hormones, the ecdysteroids, that are secreted by specific steroidogenic organs, the molting glands (Horn, 1989; Gupta, 1990). As in vertebrates, steroid biosynthesis in arthropods occurs from cholesterol and implicates cytochrome P450 enzymes (Grieneisen, 1994). Regulation of steroidogenesis in vertebrates (Baulieu, 1990) and insects (Watson et al., 1989; Smith, 1995) proceeds via a diversity of neuropeptides, adrenocorticotropic hormone (ACTH), follicle-stimulating hormone (FSH), luteinizing hormone (LH), and for insects, prothoraciotropic hormone (PTTH); all of which stimulate steroidogenesis. It is currently accepted that peptide hormones of vertebrates have dual effect on steroidogenesis given a rapid increase in steroid hormone production and a long-term effect leading to maintenance of optimal steroidogenic capacity in the steroidogenic organs (Waterman and Simpson, 1989). These two temporally separated effects were also found to be mediated by cAMP (Waterman and Simpson, 1989). The rapid response (a few min) allows the cytoplasmic cholesterol accessible to the intramitochondrial cytochromes $P450_{scc}$ to produce pregnenolone. Cycloheximide blocks the peptide-induced steroidogenesis indicating that *de novo* proteins play an essential role in this process (Privalle et al., 1983). Three classes of "regulatory" proteins operate in this rapid regulation and their synthesis is stimulated by ACTH and/or cAMP in vertebrates (Strott, 1990). Among these "regulatory" proteins are the small cytoplasmic proteins sterol carrier protein (SCP2) (Pfeifer et al., 1993) and steroidogenesis activator peptide (SAP) (Xu et al., 1991), the mitochondrial phosphoproteins belonging to the pp30-37 family proteins (Epstein and Orme-Johnson, 1991; Stocco, 1992; Clarks et al., 1994), and finally the benzodiazepine receptors (Mukhin et al., 1989; Besman et al., 1989). The long-term action of peptide hormones of vertebrates, ranging from hours to days, operates on the synthesis of steroidogenesis enzymes. These are either membrane-bound P450 cytochromes or cytosolic oxidoreductase steroids (Waterman and Simpson, 1989).

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By contrast, in crustaceans, steroidogenesis is consistently regulated by a neuropeptide, the molt inhibiting hormone (MIH),which inhibits steroidogenesis (Webster and Keller, 1989; Lachaise et al., 1993). MIH was isolated, purified, and sequenced from a neurohemal organ, the sinus gland of the crabs, *Carcinus maenas* (Webster, 1991). MIH belongs to a new family of 6–8 kDa neuropeptides that are characterized by the presence of six cysteines (Keller, 1992). Although the mechanisms of steroidogenesis inhibition in the steroidogenic organs (Y-organs) of crustaceans is still not fully understood, the three following effects can be ascertained: this inhibition by MIH is never complete; it occurs regardless of the level of steroidogenic activity (low or intense) of the tissue; it has like in vertebrates an acute and long-term effect (Webster 1991; Saïdi et al., 1994). Biosynthesis of ecdysteroids by crustacean molting glands also requires continuous synthesis of labile proteins, as it is strongly reduced by protein synthesis inhibitors (Soumoff and O'Connor, 1982; Mattson and Spaziani, 1986; Dauphin-Villemant et al., 1995). Evidence was formerly provided that cyclic nucleotides are implicated in the inhibition of steroidogenesis using sinus gland extracts (Mattson and Spaziani, 1985, 1986; Sedlmeier and Febrich, 1993) or purified MIH (Saïdi et al., 1994). These combined results suggest that cyclic nucleotides are the predominant inhibitory mediators of steroidogenic activity, and that protein synthesis plays an important role in the negative control of crustacean Y-organs. Hence, the major question that arises is whether or not inhibition of steroidogenesis by MIH occurs only by regulating the synthesis of regulatory proteins and/or enzymes like those identified in mammals, or whether it implicates other proteins?

Our purpose in this work was to look for protein(s) whose expression depends on the level of steroidogenic activity and is under the control of MIH. We analyzed the qualitative and quantitative variations of the proteic "pattern" of crab Y-organs during the molting cycle. This led us to characterize, purify, and partially sequence a 36.2-kDa Y-organ protein, which appeared to be a marker of steroidogenic activity and a target of MIH. Evidence is given that this protein is a transaldolase, and its enzymatic activity is studied relative to steroidogenesis and MIH action. The possible role of this protein that is closely related to human transaldolase is discussed.

Materials and Methods

Animals

The crabs *Carcinus maenas* were provided by the Biological Station of Roscoff (Brittany, France) and then bred as previously described in Saïdi et al. (1994). Just after molting is the period of "postmolt" corresponding to the end of elaboration of the new cuticle. The subsequent "intermolt" period is characterized by the interruption of the molting process. In this intermolt period, the level of hemolymphatic steroids is basal. In "premolt" crabs, the epidermis secretes the new cuticle and there is a high level of hemolymphatic steroids. During the molting cycle there are both quantitative and qualitative changes in the major steroids secreted: ecdysone and 25-deoxyecdysone (Lachaise et al., 1986, 1989).

Preparation of Y-Organ and MIH Incubation

Crabs were anesthetized in ice before dissection. The paired Y-organs were excised, one organ was used as control and the other exposed to MIH. Y-organs were incubated at 19°C and under N_2-O_2 (v/v) for time course studies as indicated in the appropriate figure legends. The culture medium and/or the Y-organs were collected and stored at –80°C until assayed for ecdysteroid or protein analysis, respectively.

Chemicals

Ketodiol or 2,22,25-trideoxyecdysone (107 Ci/mmol) was a gift from Dr. C. Hetru (Strasbourg, France). [^{35}S]L-methionine (1175 Ci/mmol) was purchased from Du Pont New England Nuclear and [γ^{32}P] ATP (3Ci/mmol), from Amersham (Arlington Heights, IL). Ampholytes 4–6.5 and 3–10 were obtained from Pharmacia (Uppsala, Sweden) and Servalytes 5–7 from Serva (Heidelberg, Germany). All other electrophoresis reagents were from Bio-Rad (Richmond, CA) or Sigma (St. Louis, MO). EDF duplicating and X-Omat AR films were from Kodak. cGMP was from Sigma. MIH was extracted from *Carcinus* sinus glands and purified as described in Webster (1991).

Culture Medium

Medium 199 with Hank's salts (Sigma) or medium MEM Gibco (Eagle) with Earle's salts without L-methionine and L-glutamine were adjusted to *Carcinus maenas* hemolymph osmotic pressure (1020 mosM), 0.16% m/v penicillin was added; pH 7.4. Culture medium was filtered through a 0.22 μm polyacetate membrane before use. For methionine incorporation, 200 μCi of radiolabeled methionine were added to 0.5 mL of the appropriate medium without L-methionine with 10 to 20 Y-organs depending on the size of the crab, 50 μL of sea water were added into the control medium and 50 μL of MIH (final concentration 10^{-8}*M*) into the experimental medium. Seasonal variations of steroidogenic activity of Y-organs were determined by measuring the percentage of conversion of a labeled presumed precursor: ketodiol or 2,22,25-trideoxyecdysone. One microliter of ethanol containing 1 μCi of ketodiol was added to 1 mL of medium containing from 20 to 40 Y-organs depending on the size of the crabs.

Isolation of Mitochondria

Y-organs were washed with fresh incubated medium and homogenized with 10 vol of 20 m*M* Tris-HCl buffer (pH 7.5) containing 320 m*M* sucrose, 2 m*M* EDTA, 10 m*M* EGTA, 1 m*M* PMSF and 4 μg/mL leupeptin. Homogenates were centrifuged at 1000*g* for 15 min and the supernatant was col-

lected and centrifuged at 10,000*g* for 15 min. The mitochondrial pellet was washed once in sucrose-Tris buffer and again collected by centrifugation. Mitochondrial pellets were then solubilized in a small volume of isoelectric-focusing lysis buffer (*see Preparation of Protein Samples*).

In Vitro Endogenous Phosphorylation

Organs were collected and stored at –80°C until assayed for endogenous substrate phosphorylation. The tissues were homogenized in the same buffer as for the isolation of mitochondria (*see* Isolation of Mitochondria). [γ^{32}P]-incorporation from [γ^{32}P] ATP into endogenous protein substrates was assayed in the absence or presence of a saturating level of cGMP ($5 \times 10^{-6}M$) using the same procedure as previously described (Baghdassarian-Chalaye et al., 1989). After incubation for 3 min at 30°C, the reaction was stopped by TCA precipitation and proteins were extracted for electrophoresis.

Chromatographic Separation of Radiolabeled Ecdysteroids

Y-organs and medium were mixed with acetonitrile-0.1% trifluoracetic-acid (TFA) (v/v) and sonicated. After centrifugation, 100 µL of the supernatant were injected on a reverse-phase HPLC column (Spherisorb ODS2, C18). It was eluted in an acetonitrile and 0.1% aqueous TFA non-linear gradient (30–100% acetonitrile) in 30 min.

Ecdysteroid Assays

Incubation experiments were performed with the paired Y-organs, one organ was used as control and the other as experimental. Control and MIH-treated Y-organs ($10^{-8}M$) were incubated for 1 to 20 h as indicated in the figure legend. Y-organ ecdysteroid production was measured by the EIA method described by Porcheron et al. (1989), using RUD-3 antibody (Von Gliscynsky et al., 1995) to confirm for each experiment that MIH did actually inhibit steroidogenesis.

Preparation of Protein Samples

Y-organs were homogenized, centrifuged, and stored in a lysis-buffer containing 56% (m/v) urea, 2% (m/v) CHAPS, 5% (v/v) ampholytes and 5% (v/v) β-mercaptoethanol. In order to separate proteins nonprecipitable in an acid environment, more especially, collagen—a major proteic component of the raw extract particularly abundant during low steroidogenic activity—the extraction procedure includes TCA-precipitation. Soluble proteins were thus precipitated by adding cold 10% trichloracetic-acid (TCA) to the supernatant. After centrifugation the TCA pellet was dissolved in a small volume of isoelectric-focusing lysis buffer and the solutions of total or TCA-precipitated proteins were stored at –80°C before protein estimation and electrophoresis. Protein concentration was determined by the method of Bradford modified by Ramagli and Rodriguez (1985).

Two-Dimensional Gel Electrophoresis

2D SDS-PAGE was performed following the method of O'Farrell (1975) where CHAPS is used as detergent instead of Nonidet P-40. The isoelectric point (pI) was determined by measuring the pH of 1 cm gel sections of a blank isoelectric focusing (IEF) gel; a sample containing 30 µg of protein was applied to the IEF gels. Estimates of the molecular mass of the resolved polypeptide spots were determined using their Rf and reference proteins (Low range; Bio-Rad, Richmond, CA) on the top of each slab gel. Following electrophoresis, gels were put in ethanol-acetic acid overnight, before Naphtol Blue Black, Coomassie Blue R-250, or silver staining according to Rabilloud et al. (1988). After staining, gels were dried under moderate vacuum and exposed to X-OMAT AR film for the appropriate length of time. No difference was observed between autoradiography of silver or Coomassie staining gels and this superposition permits convenient characterization of the radiolabeled proteins with [^{35}S] and [γ^{32}P].

Computer Analysis

The gels were digitized with a CCD camera and two methods were used to quantify spots on 2D gels. The computer analysis of two-dimensional electrophoresis gels was conducted with HerMès 2D computer analysis (Vincens and Tarroux, 1988) to build a synthetic 2D gel. After general image processing to remove the background and the streaks, the spots are detected and quantified. In a subsequent step, the gels were matched together to build a "master" gel by taking into account only the spots expressed at least in two of the three gels devoted to each stage of steroidogenic activity and those spots of which volumes (Vincens, 1986) were above a minimal threshold. Integration of spot volumes was performed without optical density calibration using NIH Image program (written by Wayne Rasband at the US National Institute of Health and available from the Internet by anonymous FTP from zippy.nimh.nih.gov).

Protein Sequencing

Gel spots stained with Naphtol Blue Black were cut into small pieces (not homogenized) and partially dehydrated in a SpeedVac apparatus. Gel fragments were rehydrated in 0.1*M* Tris-HCl buffer, pH 8.8, 0.03% (m/v) SDS and digested with endopeptidase Lys-C from *Lysobacter enzymogenes* (Boehringer, Mannheim, Germany) at a final concentration of 2 mg/mL for 18 h at 30°C. After centrifugation the pellet was rinsed with 60% acetonitrile and the combined supernatants were evaporated. Samples were injected onto a DEAE HPLC column linked to a C18 reverse-phase column eluted with a 0–50% acetonitrile, 0.1% TFA linear gradient. Peaks were recorded on an Applied Biosystems 1000S detector or 473 Liquid Phase Sequencer.

Table 1
Relationships Between Steroidogenic Activity (Given in % of Ketodiol Conversion), Seasons, and Molting Stages in the Crab *Carcinus maenas*

Stage	Spring	Summer	Autumn	Winter
Premolt	60–70% (H)	50–60% (H)	24–30% (M)	10–15% (M)
Postmolt	15–30% (M)	10–15% (M)	10–15% (M)	—
Intermolt	5–8% (L)	5–6% (L)	5% (L)	5% (L)

Crab Y-organs were incubated with a radiolabeled precursor of ecdysteroids(ketodiol) that is converted into two ecdysteroids: ecdysone and 25-deoxyecdysone. The proportion of ketodiol conversion (steroidogenic activity of Y-organs) varies with season and molting stage, and was used to determine the level of steroidogenic activity: (H) High, (M) Mid. (L) Low steroidogenic activity.

Computer-Assisted Identification of the P36 Protein

We used the BLAST (Basic Local Alignment Tool) program (Altschul et al., 1990) to compare the different domains of P36 protein against SwissProt protein database. The version of the algorithm was that of the GCG sequence analysis software package (Devereux et al., 1984).

Transaldolase Enzyme Activity

Y-organs or tissues were homogeneized in a buffer of 100 m*M* triethanolamine, 20 m*M* EDTA, pH 8 and centrifugated at 4°C at 10,000*g* during 15 min. Transaldolase activity was tested in the presence of 5 m*M* D-fructose 6-phosphate, 0.5 m*M* erythrose 4-phosphate, 0.15 m*M* NADH, 1.75 U/mL of α-glycerophosphate deshydrogenase and 10 U/mL of triose phosphate deshydrogenase at room temperature by continuous absorbance reading at 340 nm for 60 min (Levering and Dijkhuizen, 1990). The assay was conducted in the activity range of 0.0004–0.002 U/mL using yeast transaldolase as a control. All reagents for the transaldolase assay were from Sigma.

Statistical Analysis

Some values are expressed as mean ± SE. Statistical significance was determined by Student *t*-test. Significance was accepted when $P < 0.05$.

Results

Relationship Between Steroidogenic Activity of Y-Organs and Developmental Protein Patterns

In order to establish a scale of steroidogenic activity of Y-organs, we measured the rate of conversion of one ecdysteroid precursor (ketodiol). Combining molting stages (premolt, intermolt, postmolt) and seasons we have determined three stages of steroidogenic activity of Y-organs: a basal activity state characterized by a low ketodiol conversion range (L), a mid (M), and a high steroidogenic activity states (H) (Table 1). Y-organ proteins (at the three different levels of steroidogenic activity) were separated on 2D PAGE followed by silver staining (Fig. 1), and gels were

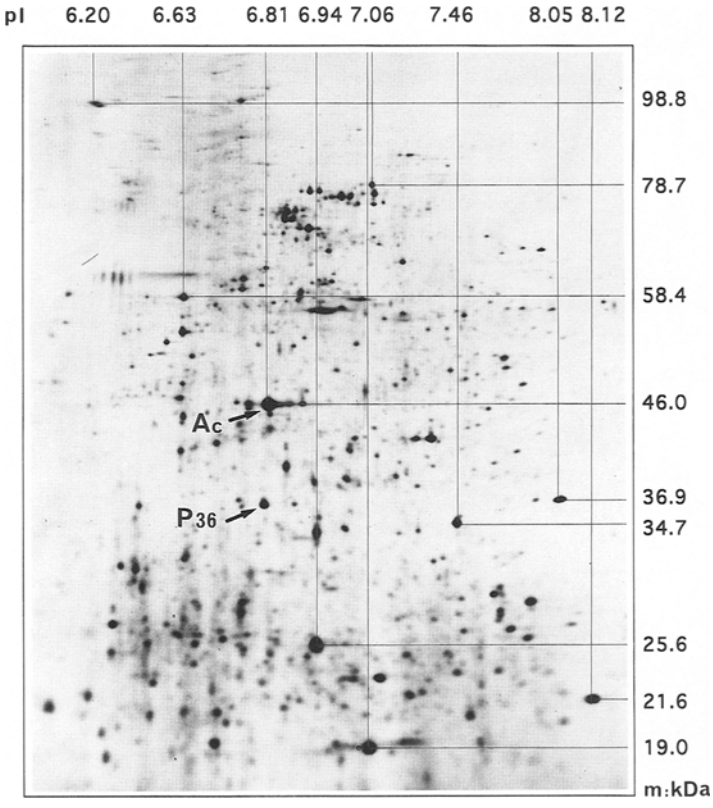


Fig. 1. Silver-stained 2D pattern of mid steroidogenic activity. TCA-precipitable proteins from crab Y-organs were analyzed using the 2D electrophoretic procedure described in Material and Methods. Molecular mass (m: kDa) and isoelectric point (pI) of internal reference proteins were determined after gel calibration. From 1000 to 1300 spots per gel were analyzed, with a pI ranging from 6–8.2 and molecular masses ranging from 18–100 kDa. Arrows indicate localization of actin spot (Ac) (molecular mass: 46; pI: 6.8) and P36 spot (P36) a major protein of Y-organ (molecular mass: 36.2; pI: 6.8).

analyzed using HerMés 2D program. The spots thereby obtained were classified in volume classes and ranked according to their number for each level of activity (Fig. 2). It can be seen that the number of spots is similar between low and mid activity (Fig. 2M and L), and in contrast, there is an increase in the number of spots of large volumes during high steroidogenic activity (Fig. 2H). The reason of the decrease in number of spots in L and M is most likely that these missing spots were precisely those with smaller volumes that were discarded from the HerMès 2D program because they were below the minimal volume threshold (*see* Material and Methods).

Actin, an Internal Reference Spot

Of interest also are a few volume classes which do not show any significant quantitative variation between L, M, and H. Among these invariant classes we retained class 21–22 (Fig. 2) and more especially a major 46-kDa spot (pI: 6.8), which quite reliably matches actin (Fig. 1). The level of this protein does not significantly vary in intensity: Measuring the volume of 10 actin spots, after silver stain-

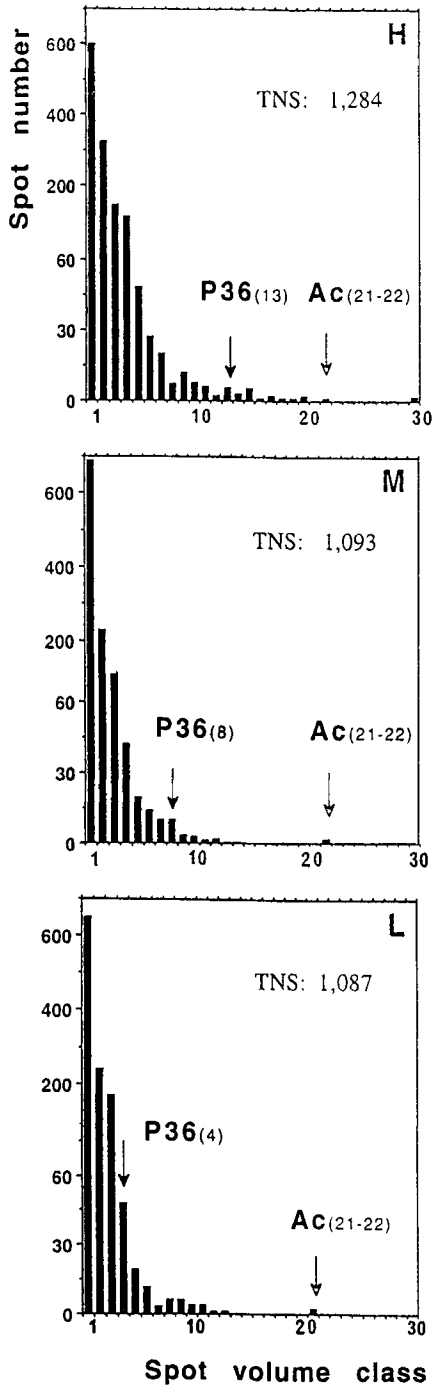


Fig. 2. Relationship between spot number and volume at high **(H)**, mid **(M)**, and low **(L)** steroidogenic activity of crab Y-organs. The silver-stained gels obtained with TCA-precipitable proteins were digitized and the volume of all spots quantified (using HerMès 2D analysis) and distributed arbitrarily in 30 vol classes. TNS: Total number of spots; (P36): P36 containing class; (Ac): actin containing class. The overall number and volume of spots shown on histogrammes in Mid **(M)** and Low **(L)** steroidogenic activity are similar. In contrast, in High activity **(H)** the overall number and volume of spots increase significantly. The volume of the spots within the actin-containing class does not significantly vary (H: 22; M: 22; L: 21), while it shifts gradually within the P36-containing class from the 13 class in high steroidogenic activity **(H)**, to the 8 class in mid activity **(M)**, and then to the 4 class in low activity. The experiments shown for high, mid, and low steroidogenic activity are representative of three.

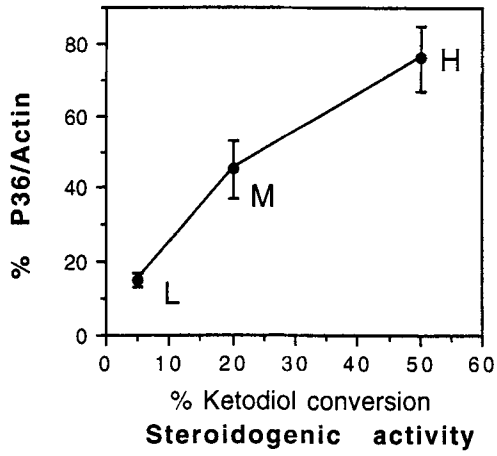


Fig. 3. Relationship between P36 expression and steroidogenic activity. After two-dimensional electrophoresis of crab Y-organs the intensity of P36 and actin spots was measured using HerMès 2D program. From "masters" gels which represent a mean of three gels for one level of steroidogenic activity (*see* Material and Methods) we determined the intensity of P36 and invariant actin spots and using this as reference we calculated the percentage of P36 during developement in Low **(L)**, Mid **(M)**, and High **(H)** steroidogenic activity (% of ketodiol conversion, *see* Table 1).

ing, and using NIH Image program, spot volumes appeared to be consistently within the range of 30,606 ± 1424 (n = 10). In order to definitely assess the identity of the protein this reference spot was isolated from the acrylamide gel to get 3 μg of pure protein. After endolysine digestion, this protein was analyzed using HPLC. One of the 37 peaks was further sequenced. The relevant amino acid sequence KCDVDIRK is common to all actins so far recorded in Genbank (Release 86, December 15, 1994). This actin spot does not vary with the steroidogenic activity of Y-organs and could, therefore, be used as internal reference.

P36: A Marker Spot of Y-Organ Steroidogenic Activity

Among the spots of which the intensity varies, the 36.2-kDa spot appeared of major interest, because of its strong relationship between its spot volume and steroidogenic activity. It is, therefore, referred here as P36 (pI: 6.8; Fig. 1), and represents 0.5% and 0.1% of the proteins under maximal and minimal activity conditions, respectively. Using HerMès 2D program, the spot intensity of P36 reaches up to 76% of that of actin when steroidogenic activity is maximal (Fig. 3); it then fits the volume 13 class (Fig. 2). With mid steroidogenic activity, the spot intensity of P36 is significantly lowered (45% of actin spot, Fig. 3) and shifts to the volume 8 class (Fig. 2). With minimal steroidogenic activity, P36 does not exceed 15% of the actin spot (Fig. 3) shifting further down to the volume 4 class of proteins (Fig. 2).

Within a given steroidogenic activity, the level of this protein does not significantly vary in intensity. Using NIH Image program and during periods of high steroidogenic activity we have measured the volume of P36 spots, they are within the range of 23,370 ± 924 (n = 10).

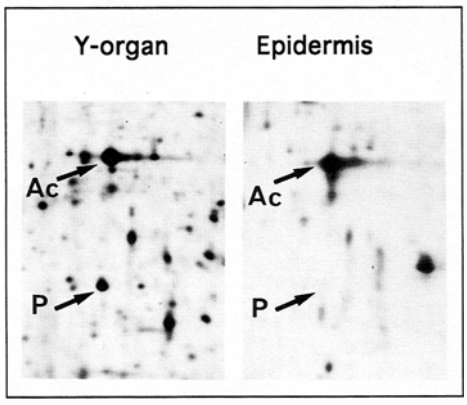


Fig. 4. Comparison of tissular intensity of P36 spot between Y-organs and epidermis: Proteins were analyzed by two-dimensional electrophoresis and after silver staining the P36 zone (36.2 kDa, pI: 6.8) was compared between epidermis and Y-organs. P: P36; Ac: Actin. No P36 protein was found on the epidermis gels.

P36 protein intensity alone can characterize the level of steroidogenic activity and we will henceforth focus our attention on this protein.

Variation of Tissular Intensity of P36 Spot

Crab Y-organs are secretory glands, of epidermic embryologic origin, riddled with blood lacunas, and surrounded by adhering tissues. So, using 2D PAGE we analyzed the protein patttern of epidermis, hemolymph, and adhering tissues and of some other tissues as brain and testis (not shown). In our current experimental procedures of electrophoresis, we did not find this protein in the epidermis (Fig. 4), hemolymph, brain, or testis (not shown). Concerning adhering tissues (not shown) P36 is present, but only as slight traces.

Subcellular Localization of P36 Protein

After removal of cellular debris and nuclei from an extract of 10 Y-organs in maximal steroidogenic activity, the mitochondrial pellets (6 μg of proteins) and their supernatants (15 μg of proteins) were analyzed independently using electrophoresis followed by silver staining (not shown). The total amount of both P36 spots (i.e., mitochondria and supernatant) closely corresponds to the spot volume expected for a total TCA extract. However, 71% of the P36 protein is found within the supernatant, which strongly suggests, bearing in mind possible contaminations, that P36 is not mitochondrial.

MIH Inhibits Incorporation of [^{35}S]Methionine into P36

We tested the effect of MIH on incorporation of [^{35}S]methionine into P36 protein in animals showing higher steroidogenic activity. Y-organs were incubated from 1, 4, 5, 6, 9, 20 h with or without MIH at $10^{-8}M$ concentration. Regardless of the length of incubation, no significant difference could be seen between P36 spot of treated and control Y-organs when gels were solely silver-stained ($t = 0.3$, $df = 8$, $P = 0.786$) (Fig. 5A). However, on autoradiogaphy

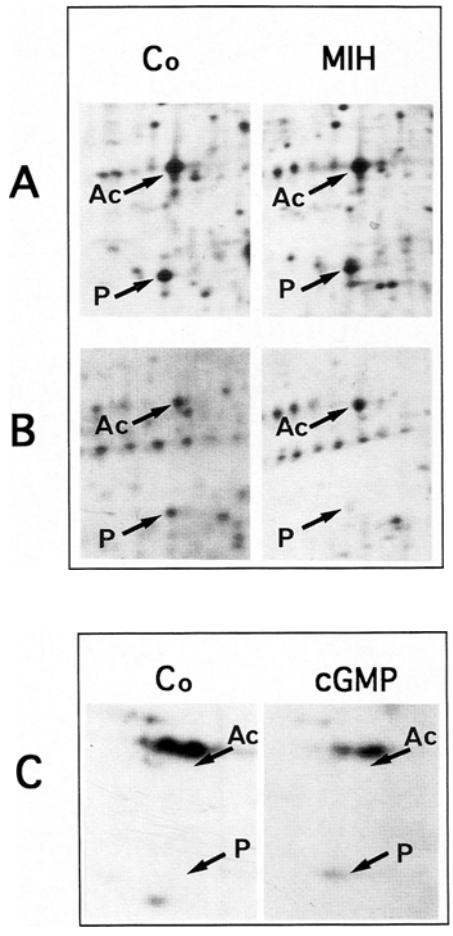


Fig. 5. Inhibition of incorporation of [^{35}S]methionine into P36 by MIH and phosphorylation of P36 protein by cyclic nucleotides. Proteins were analyzed by two-dimensional electrophoresis, after silver staining, the radiolabeled gels were exposed to X-OMAT AR film at −80°C. This superimposition permits convenient correlation between the radiolabeled proteins with [^{35}S] and [^{32}P] and silver-stained spots (*see* Material and Methods). (Ac: position of the silver-stained actin spot; P: position of the silver-stained P36 spot; **(A), (B), (C)**. **(A)** and **(B)**: Crab Y-organs in high steroidogenic activity was incubated with 400 mCi/μP [^{35}S]methionine for 9 h at room temperature, without MIH (control) and with $10^{-8}M$ MIH. After silver staining **(A)** no significant difference could be detected between control (Co) and MIH-treated organs (MIH). After autoradiography **(B)** the P36 spot of crab Y-organs incubated with MIH is significantly lowered (45%) compared to the control. **(C)** Y-organs of crab in high steroidogenic activity were homogenized and incubated for 3 min with 5 μCi of γ[^{32}P] ATP alone and with cGMP. The silver-stained gels were identical as for methionine experiments **(A)**. After autoradiography **(C)**, in control (Co) (i.e., without cyclic nucleotides) there is no spot in the P36 area while with cGMP (cGMP), a slight spot appears corresponding to P36 spot.

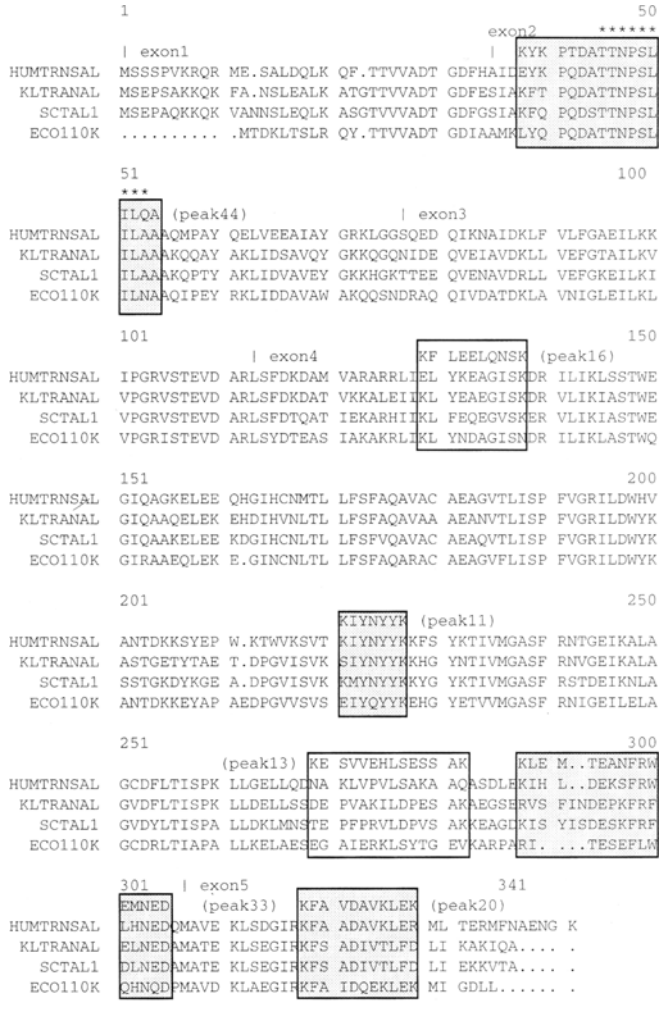
of these silver-stained gels, incubation time longer than 5- to 6-h incubation shows that incorporation of labeled methionine into P36 yielded from MIH-treated Y-organs was strongly inhibited compared to the control without MIH (Fig. 5B). The spot volume inhibition of MIH-treated [^{35}S]-P36 varies significantly from 20–55% of the control ($t = 3.9$, $df = 8$, $P = 0.005$).

Effect of Cyclic Nucleotides on ^{32}P-Incorporation Into P36 Protein

Endogenous phosphorylation was characterized by incubating Y-organ homogenates with [γ^{32}P] ATP in the absence or presence of saturating level of cGMP ($5 \times 10^{-6}M$) and autoradiographied after Coomassie or silver staining (*see* Material and Methods). Several endogenous proteins were phosphorylated. Among them, a marked spot appears above actin in control and cGMP autoradiographies (Fig. 5C). Without cyclic nucleotides, no P36 phosphorylation can be detected in the P36 area, but a spot with slightly lower molecular mass and higher acidity appears on autoradiography (Fig. 5C: Co); on cGMP-autoradiography, a slight ^{32}P incorporation into a spot which is superimposed on that of the silver-stained P36 spot on 2D-PAGE gel (Fig. 5C: cGMP).

Sequencing of P36 Protein: P36 is Closely Related to Human Transaldolase

Using 2D electrophoresis followed by slow amidoblack staining, 3 μg of P36 protein were purified from premolt Y-organs. After endolysine digestion and chromatographic separation, 45 HPLC peaks were obtained out of which six were sequenced, namely peaks 11, 13, 16, 20, 33, and 44: these are composed of 7, 14, 10, 11, 16, and 17 amino acids, respectively, representing 22% of the overall sequence of P36 protein (Fig. 6). Considering the number of peaks subsequent to endolysine digestion and of lysines characterized within the fragments, it is possible to estimate the percentage of lysines as exceeding 18%. These sequences were compared with those of a variety of proteins reported in the Genbank database. No significant homology could be detected with the following proteins involved in steroidogenesis of vertebrates, namely SCP2 (Pfeifer et al., 1993), SAP (Xu et al., 1991), StAR (Clark et al., 1994), a 30 kDa phosphoprotein belonging to a 30–37 kDa family of proteins; pI: 7.1 (Epstein and Orme-Johnson, 1991; Stocco, 1992), the 5β-reductase (Sugimoto et al., 1990), and 3α-hydroxysteroid-dehydrogenase (3α-HSD) (Cheng, 1992), two cytosolic enzymes of steroidogenesis of vertebrates, both with molecular mass about 36–38 kDa and pI around 6.8, that is close to P36 values. In contrast, there are strong homologies between P36 and four transaldolases: the human TAL-H transaldolase (Banki et al., 1994), two transaldolases of yeast, TAL-Y *Kluyveromyces lactis* (Jacoby et al., 1993), and *Saccharomyces cerevisiae* (Schaaff et al., 1990), one transaldolase of *Escherichia coli* (Yura et al., 1992) (Fig. 6). The peptide signature of transaldolases, namely TTNPSL (Reizer et al., 1995) is almost entirely contained within peak 44 sequence. In addition to the sequence homologies, these four transaldolases present a pI and molecular mass very close to those of P36 and they are cytosolic (Fig. 6). All these characteristics permit us to conclude that P36 is most closely related to the human enzyme transaldolase; TAL-C of *Carcinus maenas* was submitted to the EMBL data bank with the accession number P80427.



	Crab	HUMTRNSAL	KLTRANAL	SCTAL1	ECO110K
%	peak44	0.81	0.68	0.65	0.74
	peak11	1.00	0.89	0.89	0.85
	peak33	0.61	0.15	0.17	0.23
	peak20	0.82	0.56	0.56	0.80
pI	6.8	7.67	5.07	6.08	5.11
m:Da	36,200	37,630	36,345	36,923	35,088
	cytosol	cytosol	cytosol	cytosol	cytosol

Fig. 6. Sequence homologies between P36 and four transaldolases and biochemical characteristics. HUMTRNSAL (Human transaldolase), KLTRANAL and SCTAL1 (yeast transaldolases; *Kluyveromyces lactis*, *Saccharomyces cerevisiae*, respectively) and ECO110K (*Escherichia coli* transaldolase). Out of the six peaks sequenced in crab P36 (insets), four (44, 11, 33, 20) have great homology (dark insets) with these different transaldolases including entirely the transaldolase-typifying peak (stars). The best fit of those peaks sequenced in crab is with human transaldolase. The five regions 5' of the nucleotidic sequence exons of HUMTRNSAL are indicated. The biochemical properties of the five cytosolic transaldolases are very similar: pI: 5.07–7.67; m: 35,088–37, 630 Da.

Table 2
Tissular specificity of transaldolase

Tissues	P36 Spot	Enzymatic activity
Y-Organs (Low)	15	0.3
Y-Organs (Mid)	45	0.9
Adjacent tissues of YO	Traces	0.029
Brain	Traces	0.023
Hemolymph	Absent	0.002
Testis	Traces	0.025
Epidermis	Absent	0.007

Presence of P36 spot on 2D gels (P36 Spot, given in % of actin) and enzymatic activity (U/mg proteins) in various tissues of crab.

Transaldolase Activity

We tested transaldolase enzyme activity in a diversity of crab tissues in comparison to Y-organs. Data given in Table 2 show that transaldolase concentration in crab tissues other than Y-organs is low varying within a range from 0.002–0.029 U/mg proteins, that is values consistent with those found in vertebrate tissues (Kuhn and Brand, 1972). In Y-organs of crabs, transaldolase enzymatic activity is considerably higher than in other tissues (i.e., from 10- to 100-times as much). This transaldolase activity can reach very high values up to 1.7 U/mg proteins, a data which supports the Y-organ-high expression of transaldolase suggested above from bidimensional electrophoresis evidence (Fig. 1). In order to test whether or not transaldolase activity is related to steroidogenesis, we compared two levels of steroidogenic activity of crab Y-organs (low and mid) providing identical protein patterns (Fig. 2) and that can be found at any time of the year (Table 1). This transaldolase activity increases as a function of Y-organ steroidogenic activity (Fig. 7) and is 3 times as high in mid as in low steroidogenic activity ($t = 4.4, P = 0.0001, df = 12$). We also tested the effect of MIH on transaldolase activity of crab Y-organs in mid steroidogenic activity after 1-h or 18-h incubation (Fig. 8). After 1-h incubation with MIH, transaldolase activity is significantly decreased ($t = 3.3, P = 0.007, df = 6$). After 18-h incubation with MIH, transaldolase activity is similarly inhibited ($t = 5, P = 0.0001, df = 7$).

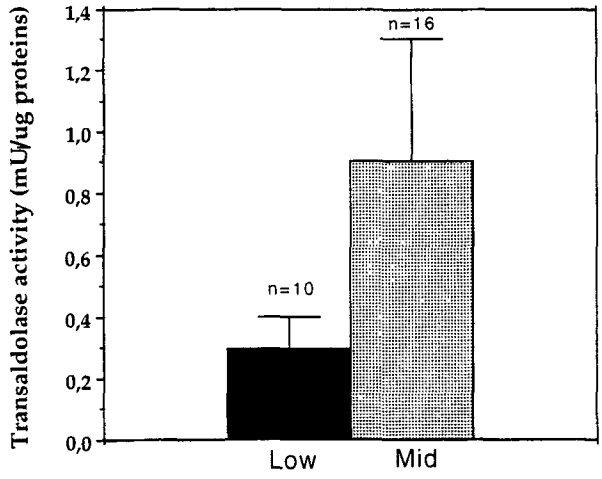


Fig. 7. Measurement of transaldolase enzymatic activity during steroidogenesis. Crab Y-organs are homogenized in TEA/EDTA buffer and steroidogenic activity is measured as in Materials and Methods. According to the stage of steroidogenic activity 1/10 or 1/30 Y-organ are used. Transaldolase activity was calculated from a reference curve with yeast transaldolase concentrations varying from 0.4–2 mU. Transaldolase activity increases from 0.3 U/mg proteins in low steroidogenic activity (Low) to 0.9 U/mg proteins in mid steroidogenic activity (Mid).

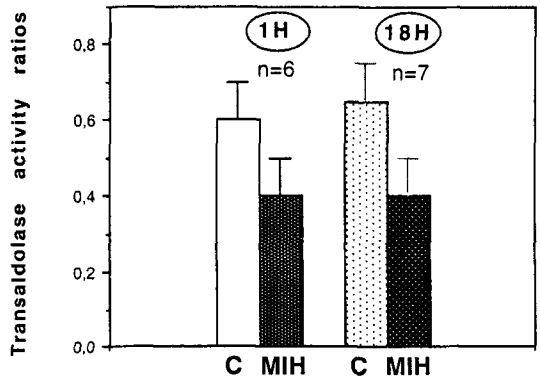


Fig. 8. Transaldolase enzyme activity with and without MIH treatment. C: control; MIH: after MIH treatment. Transaldolase activity ratios were calculated from transaldolase activities given in U/mg proteins. c: transaldolase activity of control, mih transaldolase activity of treated. Control: c/c+mih; Treated: mih/c+mih. MIH treatment decreases transaldolase activity irrespective of short (1H) or long (18H) incubation time.

Discussion

There is growing evidence that steroidogenesis regulation does not depend only on cholesterol translocation and the expression of the enzymes of the steroid biosynthesis. Other less direct types of regulation exist, acting for example, on the mitochondrial and microsomal electron transport pathways necessary to support steroid hydroxylase activities in all steroidogenic tissues. In rat adrenal gland the existence of a 43-kDa protein that retains capacity to stimulate steroid synthesis was reported (Paz et al., 1994). Moreover, a flavoprotein needed for cytochrome P450 activity was shown to be posttranscriptionally regulated by cAMP, the second messenger in hormonal stimulation of steroidogenesis (Hum and Miller, 1993). In addition, concerning the steroidogenic tissues of vertebrates, it has long been shown that gonadotropins (Mc Kerns and Ryschkewitsch, 1976) and ACTH (Criss and Mc Kerns, 1969) stimulate glucose 6-phosphate deshydrogenase (G6PDH), an enzyme of the pentose phosphate pathway (Schaaff et al., 1990). The activity of this enzyme was correlated to steroidogenic activity, and the assumption was made that G6PDH plays a role in steroidogenesis regulation through an increase of NADPH (Mc Kerns, 1966;

Bentley et al., 1990). In *Drosophila,* the activity of another enzyme of the pentose phosphate pathway, 6-phosphogluconate deshydrogenase (6-PGDH) is required for normal development. In fact, a switch off of the second reaction of the phosphate pathway by a Pgd mutation has a lethal effect: *Drosophila* larvae carrying the "zero" Pgd mutation are unable to perform the larval molt and, therefore, development is interrupted at the end of the first larval instar (Gvozdev et al., 1977). Indeed, in insects as in crustaceans the molting process is closely related to the rate of circulating ecdysteroids and hence steroidogenesis.

In this work, computer analysis of 2D electrophoresis gels of Y-organs during the three levels of steroidogenesis revealed a major cytosolic 36.2-kDa protein (P36) characterized as a transaldolase, an enzyme of the pentose phosphate pathway known to generate NADPH. This enzyme is strongly expressed in Y-organs while only detected as mere traces in other tissues. It was shown in a previous work that MIH increases the rate of intracellular cGMP in Y-organs whatever the level of steroidogenic activity may be (Saïdi et al., 1994), suggesting that cGMP is a second messenger in MIH action. A slight cGMP-dependent phosphorylation of transaldolase was observed in *Carcinus* Y-organ homogenates previously incubated in the presence of [γ ^{32}P] ATP. Moreover, we show that the enzymatic activity of transaldolase is 10- to 100-times as high in Y-organs as in any other crab tissues tested where it is within the range of the activity found in various vertebrate tissues (Kuhn and Brand, 1972). Evidence is provided that transaldolase enzymatic activity and expression increase as a function of steroidogenic activity, and were inhibited by MIH. In crustaceans, as in vertebrates, ecdysteroid biosynthesis requires cytochrome P450 that are NADPH-dependent enzymes (Grieneisen, 1994). Transaldolase, one of the enzymes of the nonoxydative part of the pentose phosphate pathway that is known to generate NADPH, plays an important role in the balance of metabolites in this pathway (Schaaff et al., 1990). Our results suggest that transaldolase, a major protein of Y-organ, could have (like G6PDH) a role in the conversion of sterols into ecdysteroids via NADPH. This set of data allows us to propose the existence in crustaceans of a steroidogenesis inhibition pathway that would proceed via transaldolase.

The question then arises as to whether or not this correlation between transaldolase and steroidogenesis can be generalized. The transaldolase genes were recently identified in bacteria (Yura et al., 1992), yeast (TAL-Y) (Jacoby et al., 1993; Schaaff et al., 1990), and human myelomonocytic leukemia cells (TAL-H) (Banki et al., 1994). These enzymes are highly conserved and present strong structural homologies. The *Carcinus* transaldolase appears nonetheless closer to the enzyme characterized in human cells. The different peptides obtained from *Carcinus maenas* transaldolase correspond to the nucleotidic sequences of TAL-H distributed over the whole coding sequence (exon 2, 4, and 5) (Fig. 6). Unlike the yeast gene which lacks introns, that of human transaldolase contains five exons distributed over a chromosomal region of ca. 50kb (Banki et al., 1994). Although the human transaldolase gene presents only one copy per genome, the region including exon 2- mid intron-exon 3 is a highly repetitive element, the TARE element, present at the rate of 1000 to 10,000 copies per haploid genome (Banki et al., 1994); it is related to retrotransposons (Finnegan, 1989) reminiscent of *copia* in *Drosophila* or *Ty* in yeast. Interestingly, the proteic sequence in *Carcinus* presents 80% homology with this region in TAL-H against 65–68% in TAL-Y (Fig. 6). No highly repetitive elements within the coding sequence of transaldolase have been reported either in yeast or in bacteria. Although the coding sequence of transaldolase is highly conserved from yeast to human, the organization of this gene appears more variable when one considers introns and/or transposable elements that might have an impact in the evolutionary process or the emergence of new functions?

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