MOLECULAR BIOLOGICAL STUDIES ON TEMPERATURE ACCLIMATION IN THE GOLDFISH

キンギョの温度馴化に関する分子生物学的研究

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Contents

Abbreviations

ATP, adenosine 5'-triphosphate

BSA, bovine serum albumin

bp, base pairs

CAPS, 3-cyclohexlamino-1-propanesulfonic acid

DAB, 3,3'-diaminobenzidine tetrahydrochloride

DTT, dithiothreitol

EDTA, ethylenediamine tetraacetic acid

HSP, heat shock protein(s)

Hx, hemopexin

IgG, Immunoglobulin G

kbp, kilo base pairs

LPS, lipopolysaccharide

MOPS, 3-(N-morpholino)propanesulfonic acid

PCR, polymerase chain reaction

PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid)

PMSF, phenylmethanesulfonyl fluoride

PVDF, polyvinylidene difluoride

RNApase, RNA polymerase

RT-PCR, reverse transcription-PCR

SDS, sodium dodecyl sulfate

SDS-PAGE, SDS-polyacrylamide gel electrophoresis

TFA, trifluoroacetic acid

TH, tyrosine hydroxylase

TBS, Tris-buffered saline

Wap65, warm acclimation-related 65-kDa protein

wap65, Wap65 encoding gene

Introduction

Temperature is one of the environmental factors which has an extremely pervasive effect on the cellular and molecular functioning of poikilotherms, as body temperature must stabilize closely to that of the external environment. Apart from endothermic fish, all fish belonging to the vertebrate class Agnatha, Chondrichthyes, and Osteichthyes are ectothermic.

Although changes in body and ambient temperature are obligately linked, fish manage and compensate for thermal stress by variety of mechanisms. These include, (1) behavioral adjustments; (2) physiological or autonomical responses to acute temperature change; (3) acclimatory responses to seasonally altered temperatures within the life time of an individual; and (4) adaptational adjustment to persistently altered temperature over evolutionary time (Hazel and Prosser, 1974). Whereas first two of these mechanisms are complementary and activated within seconds to minutes, the third requires some period of exposure to altered body temperatures before becoming effective. The fourth require much time to result in genotypic change. This temporal hierarchy of compensatory response provides a conceptual framework for discussing the thermal biology of poikilothrmic fish.

Among these four types of compensation, the present study focused on the acclimatory response from a view point of gene expression regulated by ambient temperature. Although, a

large number of classical evidence for thermal acclimation in fishs and other poikilotherms has been extensively reviewed (Hazel and Prosser, 1974; Hochachka and Somero, 1984), selected evidences will be used to illustrate the phenomena.

When a poikilotherm is transferred from one thermal regime to another, adaptive changes are initiated that allow the organisms to maintain a similar rate of physiological activity, such as performance, neural function, oxygen consumption and so on (Hazel and Prosser, 1974). This compensation for temperature fluctuations is often referred to as temperature acclimation.

Thermal acclimation in fish is most evident in eurythermal species such as goldfish and carp that must accommodate to seasonal fluctuations in temperature. Individual fish are able to survive at widely different water temperature ranging between near 0 to 30°C. Acclimation to temperature extremes both improves performance at the acclimation temperature and extend the range over which normal activity can be maintained. For instance, in goldfish, the upper lethal temperature is increased 1°C (from 31 to 41°C) for every 3°C rise in acclimation temperature (from 10 to 37°C) (Fry, 1971).

Temperature acclimation is also evident at the level of whole animal and cellular metabolism. An individual of a species displays higher rate of oxygen consumption when acclimated to cold than warm. For example, goldfish acclimated to 10°C exhibits rates of standard oxygen consumption which are 1.92-fold higher than that of fish

acclimated to 30°C when compared at an intermediate temperature of 15°C (Kanugo and Prosser, 1959). At the cellular level, the capacity for ATP production by aerobic mechanism is commonly elevated at cold temperature, whereas enzymes associated with lysosome and nitrogen metabolism are elevated at warm temperature (Hazel and Prosser, 1974). It is noted that the changes induced by temperature acclimation are derived from comparisons between individuals of the same species that have been acclimated to different temperature. In many cases, warm and cold temperature acclimation indicate the same change from different view points. For example, enhanced rate of oxygen consumption may be adaptive to maintain fish life in the cold water. Conversely, reduced rate of that may also adaptive for fish at warm water temperature to regulate proper metabolism.

The concept extends beyond acclimation of membrane fluidity. Basolateral membrane of enterocytes from 10°C-acclimated carp is more fluid compared with that from 30°C-acclimated fish when measured at a common temperature (Hazel and Williams, 1990). The change may be functional to both acclimated fish because the fluidity are roughly equivalent when measured at the respective body temperature.

Since those adaptations require a period of time that may last from days to weeks, biochemical reorganization resulted from altered gene expression by ambient temperature is apparently involved.

Temperature regulation of gene expression is clearly illustrated by the synthesis of heat shock proteins, a highly

conserved family of stress protein (Near *et al.*, 1990). Heat shock proteins are transiently synthesized during acute heat stress and their synthesis permit a cell transiently to tolerate subsequent exposure to temperature that could otherwise be lethal (Pelham, 1986). Since the synthesis of heat shock proteins require a few hours and shows transient increase (Mosser and Bols, 1988), the response to acute heat should be distinguished from relatively long-term adjustment of thermal acclimation in terms of time scale and the range of temperature fluctuation. In the classical frame work of temperature adaptation mentioned at the beginning of this chapter, the heat shock response is out of phase. It is proper that the second category of responses to acute temperature change should be expanded and connote this cellular event.

The seasonal appearance of antifreeze proteins in some marine teleosts is a rare example of phenomena fosused on gene expression correlated to temperature acclimation (Chen *et al.*, 1993). These peptides lower the plasma freezing point noncolligatively and allow the fish to survive low temperatures (Davies and Hew, 1990). Although these peptides are obviously responsible for cold acclimation in icy seawater, photoperiod rather than temperature is the primary factor regulation the expression of antifreeze protein gene in winter flounder (Flecher *et al.*, 1989; Vaisius *et al.*, 1989; Fourney *et al.*, 1984). The production of these peptides are a strategy of coping with extremes of environmental temperature and seems not adopted by many fish species.

Although many physiological and biochemical evidences for thermal acclimation of eurythermic temperate fish have been described, few studies have focused on gene expression associated with temperature acclimation in spite of its significance to understand those divergent knowledge from a convergent and unified perspective.

From these background studies, the following themes can be raised as necessary to elucidate in the field of fish temperature acclimation: presence of genes of which expression is regulated by acclimation temperature; primary structure, functions, and localization of their translated products; and regulatory mechanisms of such gene expression. The final goal in such a course of investigation is to identify all member of thermally regulated gene and to elucidate physiological roles of such genes together with their organized regulation. However, the last-mentioned aspect is beyond the scope of this thesis.

Goldfish, *Carasssius auratus*, has been used for studies on temperature acclimation because of its eurythermic ability and appropriate size as a laboratory material. In addition, investigations dealing with acclimatory response at the physiological and biochemical levels have been accumulated (Hazel, 1993). Thus, goldfish is considered as a good experimental model for the study of temperature acclimation at the molecular level.

This thesis consists of five chapters. Chapter 1 deals with changes in the protein composition of goldfish by temperature acclimation. The presence of proteins associated

with temperature acclimation have been confirmed.

In Chapter 2, the warm temperature acclimation-related 65-kDa protein (Wap65) in goldfish is discussed. The changing levels of Wap65 following temperature acclimation and wide distribution of Wap65 in various tissues are also examined using a specific antibody in Chapter 2. Chapter 3 deals with the purification of Wap65 from goldfish muscle and N-terminal amino acid analysis. Features of the primary structure of Wap65, which were deduced from cloned cDNA, are discussed in Chapter 4. Chapter 5 deals with gene expression of Wap65 in the process of temperature acclimation. Temperature has been demonstrated to influence the transcript level of Wap65. Tissue-specific expression of this gene are also examined. Subsequently, deduced promoter region of Wap65 gene are analyzed to comprehend the regulatory mechanisms of Wap65 gene expression.

Chapter 1

Changes in protein composition in goldfish due to temperature acclimation

Seasonal acclimatization of fish involves compensatory responses to fluctuation of ambient temperature. Many of the physiological adjustment responsible thermal acclimation appear to result from altered gene expression which is interpreted in the broadest sense to include modulation of transcription, translation, post translational modification, or protein catabolism.

One of the best grounds for the supposition is the changes in enzymatic activity and cellular metabolism induced by temperature acclimation. The large number of enzymes exhibit changes in catalytic activity during thermal acclimation irrespective of temperature-dependancy of chemical reaction (Hazel and Prosser, 1974). For example, catabolisms of carbohydrate via pentose phosphate pathway and the Krebs cycle is generally elevated in cold-acclimated fishes in comparison to warm-acclimated fishes (Helly, 1976). But the rate of glycolysis does not show an elavation (Jones and Sidell, 1982).

These enzymatic alterations may be attributable to altered gene expression including quantitative alterations of enzyme synthesis, qualitative changes in isozyme expressions, or changes in microenvironment of enzyme such as lipid composition of cellular membranes (Hazel and Posser, 1974). However, few studies focused on gene expression associated

with temperature acclimation.

Since the principle of gene expression is a base of biological phenomena, molecular biology of temperature acclimation in fish provide a convergent viewpoint to understand divergent observations previously reported in the field of fish temperature acclimation. It is important to focus on the synthesis of a particular protein which behaves in response to ambient temperature, to understand the acclimatory mechanisms at the level of gene expression.

To examine the presence of candidate proteins which can facilitate research on the molecular biology of temperature acclimation in fish, changes in protein compositions of goldfish acclimated either 10 or 30°C were investigated. This chapter presents the result of these investigations.

Materials and Methods

Materials

Goldfish *Carassius auratus* (36 - 45 g) were acclimated to either 10 or 30°C for a minimum of five weeks in laboratory aquariums. The acclimation period was determined in reference to the data of Heap *et al.* (1985) for carp. All fish were fed commercial pellets daily ad libitum. After acclimation, fish were sacrificed and white epaxial muscle, hepatopancreas, and brain were dissected out. In order to compare protein composition patterns between cold- and warm-acclimated goldfish, muscle, hepatopancreas, brain and plasma

from 16, 10, 10 and 6 individuals, respectively, for each of the two acclimation groups were subjected to electrophoretic analyses. In the case of blood sampling, fish acclimated for seven weeks were used.

Preparation of tissue extracts

Muscle and hepatopancreas were homogenized in an equal volume of an ice-cold 1:1 (v/v) mixture of 0.06 M barbital buffer (pH 8.6) and glycerol. The two homogenates were centrifuged at 33,000 x g for 15 min and the resulting supernatants were used for electrophoretic analyses. In the case of brain, 4 volumes of the same buffer was added to homogenize tissues.

Blood sampling

Approximately 0.05 ml of blood was drawn from the caudal vasculature with a heparinized syringe fitted with a No. 23-gauge needle after anesthetizing fish with 600 ppm of 2-phenoxyethanol (Wako). Blood samples were centrifuged at 3,000 rpm, and plasma stored at -20°C until electrophoretic analysis.

Electrophoretic analyses

SDS-PAGE was performed by the method of Laemmli (1970) using 7.5 - 20% polyacrylamide gradient slab gels containing 0.1% SDS. Two-dimensional electrophoresis was performed by the method of O'Farrell (1975) using 4% polyacrylamide gels in the presence of 8 M urea and 1% Ampholine (comprised of 0.8%

pH range 5 - 8 and 0.2% pH range 3.5 - 10) for isoelectric focusing and 12% slab gels for SDS-PAGE. Gels were stained with 0.1% Coomassie Brilliant Blue R250 after conventional one-dimensional SDS-PAGE, or with silver nitrate after two-dimensional electrophoresis according to Oakley et al (1980). For SDS-PAGE, tissue extracts were treated with the same volume of the sample buffer containing 20 mM Tris-HCl (pH 6.8), 2% SDS, 40% glycerol, 2% β-mercaptoethanol, 4 mM ethylenediamine tetraacetic acid (EDTA) and 0.001% bromophenol blue. After incubation at 100°C for 3 min, 20 µl of the sample were subjected to electrophoresis. In the case of blood, 9 volumes of the sample buffer were added to the plasma, and 3 µl of the sample were used following incubation at 100°C for 3 min. For isoelectric focusing, tissue extracts were treated with the same volume of isoelectric focusing solution containing 8 M urea, 10% NP-40 and 1% Ampholine comprised as mentioned above. The sample volumes applied were 8 µl. In the case of plasma, the isoelectric focusing solution was added to 3 µl of plasma upto 100 µl, and 100 µl of the sample was applied to isoelectric focusing.

Standard molecular weight markers (Sigma) were myosin heavy chain from rabbit muscle (205,000=205 kDa), β-galactosidase from *E. coli* (116 kDa), phosphorylase b from rabbit muscle (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase from bovine erythrocytes (29 kDa).

Results

Proteins from the muscle

Two components were found to be predominant in goldfish muscles by one-dimensional SDS-PAGE followed by Coomassie Brilliant Blue staining (Fig. 1-1). They had molecular masses of approximately 45 kDa and 12 kDa and were regarded as creatine kinase and parvalbumin, respectively, according to Nakagawa *et al.* (1988) and Hamoir (1974). However, differences were hardly observed in electrophoretic patterns between the 10°C- and 30°C-acclimated fish except for a 65-kDa protein which was abundant in the 30°C-acclimated goldfish.

When extracts from the muscle were subjected to two-dimensional electrophoresis and subsequently stained by a more sensitive method with silver nitrate, goldfish exhibited variations according to their acclimation temperatures in the relative abundances of 65- and 55-kDa protein components. Figure 1-2 shows typical patterns from 16 individuals each for the two groups of acclimated goldfish. Besides the large quantities of creatine kinase located in the center of the gels, the 30°C-acclimated goldfish showed increased abundance of the 65-kDa protein, whereas the 10°C-acclimated one showed increases in the 55-kDa protein. It is noted that both proteins were extremely acidic. Thus far, no sex-dependent variations have been observed in goldfish.

Proteins from the hepatopancreas

The same analytical methods as those adopted for muscle

were used for proteins from the hepatopancreas. One-dimensional SDS-PAGE showed that 97- and 28-kDa proteins increased remarkably following 30°C acclimation (Fig. 1-3). Other proteins were roughly constant irrespective of acclimation temperatures, except for some proteins with molecular masses of approximately 45 kDa which were abundant, though with individual variation, in the 10°C-acclimated hepatopancreas. It is noted that a 120-kDa component expressed in the 10°C-acclimated hepatopancreas was found only in female fish.

Two-dimensional electrophoretic patterns of hepatopancreas soluble proteins were less clear than those of muscle proteins, probably due to contaminating lipids (Fig. 1-4). However, it was demonstrated that the 65-kDa protein was again increased in the hepatopancreas by 30°C acclimation as in the case of muscle tissues. The increased abundance of the 28-kDa protein in the 30°C-acclimated goldfish was also observed in the two-dimensional electrophoretic gel.

Proteins from the brain

SDS-PAGE patterns of brain tissue extracts showed the increased abundance of components with molecular masses of 28 kDa and more than 200 kDa following 30°C acclimation (Fig. 1-5). On two-dimensional electrophoretic gels stained with silver nitrate, protein profiles were similar to those of the muscle and hepatopancreas extracts, showing an acclimation temperature-dependent change with respect to the 55- and 65-kDa components (Fig. 1-6). These results suggest that the two

acidic components having molecular masses of 65 and 55 kDa are ubiquitous proteins the quantities of which are changed in response to temperature acclimation of goldfish.

Plasma proteins

Electrophoretic analysis of plasma proteins revealed increased abundance of the 65-kDa protein as in the case of various tissue extracts (Fig. 1-7 and 1-8). Though individual variations were observed, other proteins were independent of acclimation temperatures except for a 28-kDa protein. The 28-kDa protein seemed to be slightly abundant in the warm temperature-acclimated fish by SDS-PAGE (Fig. 1-7). On two-dimensional electrophoresis gels, cold-acclimated fish expressed a 28-kDa protein that have alkalescence isoelectric point compared with major 28-kDa protein (Fig. 1-8).

Discussion

The two-dimensional electrophoretic analyses of soluble proteins from the muscle, hepatopancreas, and brain disclosed that the 55- and 65-kDa protein components increased in their quantities during 10 and 30°C-acclimation, respectively. In addition, increased abundance of the 65-kDa protein was also observed in plasma from the 30°C-acclimated fish. These results led to the speculation that one of adaptive strategies in temperate freshwater fish in response to environmental temperature changes is *via* quantitative alterations of

temperature acclimation-associated proteins. However, the relevance of such alterations to their protective effect against thermal stress is not clear at the present stage. To answer these questions, it seems necessary to isolate the proteins concerned and characterize them.

Two-dimensional electrophoresis in this study revealed that the 65-kDa protein was increased in brain, muscle, hepatopancreas, and plasma following 30°C-acclimation. Since the 65-kDa protein seemed to be one of major components in plasma, a possibility for the accumulation of the 65-kDa protein in those tissues due to blood flow-through remained. This will be discussed again in Chapters 4 and 5. The proteins which may be observed simultaneously in several tissues at moderately high acclimation temperature of 30°C have not been reported, at least to the author's knowledge. In addition, it is evident that the 28-kDa protein as well as the 65-kDa protein increased in hepatopancreas and brain tissue extracts from the 30°C-acclimated goldfish (see Figs. 1-3 and 1-5). It has been reported that the synthesis of an apolipoprotein having a molecular mass of 29 kDa was enhanced in isolated hepatocytes from carp acclimated to summer temperatures (Instroza *et al.*, 1990). However, the relationship of the 29-kDa protein in carp hepatocytes with the 28-kDa protein in goldfish remained uncertain. So far, no information seems to be available on the function of proteins specific to 10°C-temperature acclimation such as the 55-kDa component found in this study.

On the contrary, many studies concerning heat stress on

cytosolic protein profiles have been carried out. Thermotolerance is acquired in animals which have been previously exposed to acutely elevated temperatures, in other words subjected to the well-known "heat shock" (Shimada *et al.*, 1985). It is interesting that poikilothermic organisms inhabiting ecologically hot areas, such as desert easily produce heat shock protein (HSP) 70-like proteins having a molecular mass of about 70 kDa (Ulmazov et al., 1992). HSP70 is one of the most intensively investigated HSPs and is known to be induced in organism as diverse as from human to bacteria. Induction of HSP synthesis was also evident in teleosts including the rainbow trout *Salmo gairdnerii* (Kothary and Canolido, 1981), channel catfish *Ictalurus punctatus* (Koban *et al.*, 1987) and carp *Cyprinus carpio* (Ku and Chen, 1990). The 65-kDa protein produced in goldfish after 30°C-acclimation in this study was similar to HSP70 proteins in terms of isoelectric point, molecular weight and wide distribution to various tissues. The 65-kDa component showed no cross-reaction with monoclonal antibody against 72-kDa HSP from HeLa cells (Amersham) in western blotting (data not shown). However, it is still ambiguous whether or not the 65-kDa protein found in this study belongs to a family of HSP70 proteins. Therefore, it seems necessary to determine the amino acid sequence of the present 65-kDa protein, since HSP70 proteins are highly conserved in their sequences (Lindquist, 1986). Such investigations are dealt in Chapter 2.

Chapter 2

Specification of the warm temperature acclimation-related 65-kDa protein, Wap65, in goldfish

In Chapter 1, the changes in protein composition of goldfish acclimated to either 10 or 30°C were investigated, revealing the increased abundance of the 65-kDa protein in several tissues in 30°C-acclimated fish. Thus, ubiquitous nature together with a potential function of this component in the acclimatory response of goldfish were postulated.

Investigations in Chapter 1 also suggested the similarity of the 65-kDa protein to HSP70 in terms of isoelectric point, molecular weight and wide distribution in various tissues. HSP70 is one of heat shock protiens which are synthesized immediately but transiently in cells exposed to elevated temperatures (Lindquist, 1986). The HSP70 family members are the most highly conserved in their structure and phylogenetically ubiquitous among HSPs (Near *et al.*, 1990). ATP binding property of HSP70 is also maintained in diverse organisms including teleosts (Fader *et al.*, 1994; Margulis *et al.*, 1989). The synthesis of HSP70 directly correlates with the acquisition of thermotolerance (Pelham, 1986). Beside the instantaneous temperature compensation including the synthesis of HSPs at the cellular level, compensation is evident at the level of individual after a relatively long period of temperature acclimation, usually a few days or weeks in duration. For example, myofibrillar ATPase activity is

increased in goldfish after cold acclimation (Johnston *et al.*, 1975). This increase is manifest after 1 or 2 weeks and enzyme activity reaches a steady state after 4 or 5 weeks for carp (Heap *et al.*, 1985). In general, such acclimatory response is maintained at least several weeks to compensate for seasonal variations in environmental temperature (Hazel and Prosser, 1976).

In this study, the goldfish 65-kDa protein was isolated from two-dimensional electrophoretic gels and its antibody was raised to carry out immunoblot demonstrations. An ubiquitous tissue distribution of the 65-kDa protein and its time course for increasing concentrations in the muscle during warm temperature acclimation were examined by immunoblot analysis. An affinity chromatography for muscle extracts followed by immunoblot analysis was performed to examine ATP-binding property which is regarded as one of the characteristic properties of HSP70 proteins. An N-terminal amino acid sequence was also determined for comparison with those sequences of the HSP70 proteins.

Materials and Methods

Fish

Goldfish (36 - 45 g) were acclimated to either 10 or 30°C for five weeks in laboratory aquariums as described in Chapter 1. After acclimation periods, 10 individuals each from 10 and 30°C temperatures were killed while not struggling and samples

of white epaxial muscle, hepatopancreas and brain were dissected.

The effects of raising water temperature from 20 to 30°C on protein composition were examined in 20 individuals (1.5 - 5.5 g) acclimated at 20°C for five weeks. The 20°C-acclimated fish were subjected to increased temperature to 30°C in an aquarium of 60 x 40 x 30 cm with a thermocontroller (Toho, TP-173) over a 20 h period. After 4 h resting at 30°C, 5 fish were taken out from the aquarium to analyze concentrations of the 65-kDa component in extract of epaxial muscle. Other groups each containing 5 sample fish were taken out periodically from the 30°C-aquarium over a 10 day duration to examine the changes in the 65-kDa protein concentrations during acclimation (see Fig. 2-6).

Preparation of tissue extracts

Muscle and hepatopancreas tissues were homogenized in an equal volume of an ice-cold 1:1 mixture of 0.06 M barbital buffer (pH 8.6) and glycerol (v/v) and centrifuged at 33,000 x g for 15 min as described in Chapter 1. The resulting supernatants were used for electrophoretic analyses. The muscle extract was also subjected to ATP-agarose affinity chromatography. In the case of brain, 4 volumes of the same buffer were added to homogenize tissues.

Blood sampling

Blood sampling was carried out as described in Chapter

1.

Electrophoretic analyses

SDS-PAGE and two-dimensional electrophoresis were performed as described in Chapter 1. Sample volumes used were 20 µl for one-dimensional SDS-PAGE. On two-dimensional electrophoresis, 8 and 200 µl lysate were used for immunoblot analysis and protein isolation, respectively.

Antibody preparation

Muscle extracts were obtained from the goldfish acclimated to 30°C. Extracts were subjected to two-dimensional electrophoresis, after which the 65-kDa protein was extracted with 70% formic acid for 2-3 days at 4°C from gels stained with Coomassie Brilliant Blue (Kobayashi *et al.*, 1985). Antibody against the 65-kDa protein was raised in rabbit using about 200 µg of antigen by conventional methods. The IgG fraction was purified from the antisera by using a protein A-Sepharose affinity column (1.6 x 4.0 cm, Pharmacia).

Immunoblot analysis

Immunoblot analysis was performed on an polyvinylidene difluoride (PVDF) membrane (Immobilon, Millipore) after electrophoresis using the antibody against the 65-kDa component. Proteins on SDS-PAGE gels were electrophoretically transferred to a PVDF membrane according to the method of Towbin *et al.* (1979) using a blotting buffer containing 25 mM Tris, 192 mM glycine, 20% methanol and 0.02% SDS.

Subsequently, the membrane was incubated in Tris-buffered saline (TBS) consisting of 50 mM Tris-HCl (pH 8.0) and 0.9% NaCl for 15 min, in TBS containing 2% BSA for 1 h, in TBS containing 0.2 or 0.6% BSA and antibodies against the goldfish 65-kDa protein for 1 h, and washed 3 times in TBS for 5 min. Biotin labeling of IgG and immunostaining of the membrane were performed using the Vectastain Avidin-Biotin Complex kit according to an appended manual (Vecter Lab.). Briefly, the membrane was incubated in TBS containing 0.2% BSA and avidin-peroxidase complex for 1 h. After 3 times washing in TBS, reacting protein bands were detected by addition of 50 ml of TBS containing 25 mg of 3,3'-diaminobenzidine tetrahydrochloride (DAB) and 10 µl of 31% hydrogen peroxide. After saturating the membrane in DAB solution, staining was enhanced with the addition of 50 µl each of $CoCl_2$ and $NiCl_2$ at 200 mM.

N-terminal amino acid sequencing

The N-terminal amino acid sequence was determined by the method of Matsudaira (1987) as follows. Proteins of muscle extract separated on SDS-PAGE were electrically transferred onto an Immobilon PVDF membrane using blotting buffer containing 3-cyclohexlamino-1-propanesulfonic acid (CAPS) for eliminating a possible contamination of glycine and stained with Coomassie Brilliant Blue R250. The part of the membrane carrying the blotted protein was cut out with a clean razor. Several membranes were placed together on the teflon seal of the cartridge block in an Applied Biosystems model 477A

protein sequencer with an on-line system model 120A.

ATP-agarose affinity chromatography

ATP-agarose affinity chromatography was performed according to Welch and Feramisco (1985) with slight modifications. Briefly, the muscle extract obtained by the same method of electrophoretic analysis was dialyzed against 20 mM Tris-acetate (pH 7.5) containing 20 mM NaCl, 3 mM MgCl, 15 mM β-mercaptoethanol, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.05% NaN_3 (buffer A). The sample was then applied to an ATP-agarose column (Pharmacia, 1.2 x 5.0 cm) and the column was washed with buffer A containing 0.5 M NaCl followed by buffer A alone. The proteins absorbed were then eluted with buffer A containing 3 mM ATP. A flow rate was fixed at 1 ml/min throughout and 3 ml fractions were collected. The elute was monitored with absorbance at 280 nm. The muscle extract and fractions were analyzed for their protein compositions by one-dimensional SDS-PAGE in 12.5% polyacrylamide gels and subsequent immunoblot analysis.

Results

Two-dimensional electrophoretic pattern

When proteins from muscle were subjected to two-dimensional electrophoresis and subsequently stained with Coomassie Brilliant Blue, the 30°C-acclimated goldfish showed

an increased abundance of the 65-kDa protein component as previously reported in Chapter 1 (Fig. 2-1). After the separation on the gels, the 65-kDa component was subjected to extraction for raising antibody and N-terminal amino acid analysis.

Immunoblot analysis for extracts from various tissues and plasma

To examine the specificity of the antibody raised against the 65-kDa protein, immunoblot analysis was performed on an Immobilon PVDF membrane after two-dimensional electrophoresis of muscle tissue extracts from goldfish acclimated to either 10 or 30°C (Fig. 2-2). Figure 2-2 shows the immunoblot pattern with muscle extracts from the 30°C-acclimated goldfish. A strong reactivity was observed with one band corresponding to the 65-kDa protein on the membrane. No other proteins reacted with the antibody, demonstrating that the antibody has a high specificity for the protein concerned. As shown in Fig. 2-2, reaction was hardly detected on the immunoblot membrane for the 10°C-acclimated fish.

Addition of 0.5 mM protease inhibitors such as antipain, aprotinin, chymostatin, pepstatin, PMSF, trpsin inhibitor (Sigma) and dithiothreitol (DTT) did not change the molecular weight and isoelectric point of the 65-kDa protein (date not shown). Figure 2-3 shows immunoblot patterns of brain tissue extracts from goldfish acclimated to either 30 or 10°C. The quantitative differences of the 65-kDa component between the 30- and 10°C-acclimated fish were not clearly defined in one-

dimensional SDS-PAGE stained with Coomassie Brilliant Blue (see Fig. 1-3 in Chapter 1). However, strong reactions were observed on the blotted membrane after SDS-PAGE with the brain tissue extracts from the 30°C-temperature acclimated fish (Fig. 2-3, lanes 1 - 5). On the other hand, no reaction was detected in the brain tissue extracts from the 10°C-acclimated fish (Fig. 2-3, lanes 6 - 10). These results suggest that the muscle 65-kDa component has structural homology with that from brain tissues. Figure 2-4 shows immunoblott patterns of hepatopancrease extracts. Though several bands exhibited reaction with the antibody, reactive protein bands having a molecular mass of 65 kDa were specific to the 30°C-acclimated goldfish. In the case of plasma, the accumulation of the 65-kDa component was also demonstrated in the 30°C-acclimated goldfish (Fig. 2-5). These results suggest that the 65-kDa component is a ubiquitous protein.

Several bands reacting with antibody were observed on immunoblott analysis of hepatopancreas and plasma, implying that some parts of these components contain the structures similar to those of the 65-kDa protein from muscle.

Change in the 65-kDa protein concentration during the acclimation process

Water temperature of the aquarium was increased from 20 to 30°C within 20 h according to the time schedules shown in Fig. 2-6A. Figure 2-6B shows representative results obtained with 5 individuals at each acclimation condition. Although many faint bands appeared with immunostaining, the following

tendency was clearly ascertained. The 65-kDa protein concentration in the muscle extract was very low immediately after water temperature reached 30°C. However, a prominent amount of the 65-kDa protein was visualized with the specific antibody in the muscle tissue extract within 5 days after raising water temperature to 30°C. It is noted that, when the muscle extracts of 20°C-acclimated fish were examined as controls by immunoblot analysis, a strongly reacting band with a molecular mass of 70 kDa was unexpectedly observed on the membrane. The corresponding component was not found in the muscle extracts from the 10 and 30°C-acclimated goldfish. Immediately after water temperature was raised to 30°C, the 70-kDa protein increased its abundancy in the muscle and maintained a high concentration within at least 2 days, while it almost disappeared after 5 days at 30°C. Other minor faint bands observed by immunoblot analysis seemed to reflect non-specific reaction, since the incubation of membranes in TBS containing the antibodies with 0.6% BSA markedly decreased their staining intensity (see Fig. 2-8B, lane 1).

N-Terminal amino acid sequence

The N-terminal amino acid sequence of the 65-kDa protein is shown in Fig. 2-7. In total, 10 residues were determined by microanalysis and the sequence was rich in glutamine. No further analysis was possible due to the limited amount of the 65-kDa protein on the membrane. Since histidine and aspartic acid were found in about an equal amount at position 8 from the N-terminus, it is possible that the preparation of this

65-kDa protein comprises multiple components with essentially the same amino acid sequence.

ATP-agarose affinity chromatography

Figure 2-8 shows an elution profile on ATP-agarose affinity chromatography of the muscle extract from the 30°C-acclimated goldfish. The muscle extract contained various components including the major components creatine kinase, with a molecular mass of about 45 kDa (Nakagawa et al. 1988)(lane 1 of panel A in Fig. 2-6, see also Fig. 2-1). A series of washing, first with buffer A alone and second with buffer A containing 0.5 M NaCl, removed most proteins from the column. The 65-kDa component indicated with an asterisk in Panel A was also unabsorbed to the column, which was further confirmed by immunoblot analysis as shown in Panel B. In contrast, a 70-kDa component together with a 200-kDa protein was absorbed to the column and eluted with the buffer A containing 3 mM ATP. High absorbance of this fraction at 280 nm must be due to the absorbance of ATP added to buffer A for eluting ATP-binding proteins. The 70-kDa protein appeared to be a member of HSP70 in terms of the molecular weight and ATP-binding property. It is noted that the 70-kDa protein did not react with the antibody raised against the 65-kDa protein.

Discussion

In Chapter 1, it has been described that the 65-kDa protein increased in quantity following warm temperature

acclimation in the muscle of goldfish. In this chapter, an antibody against the 65-kDa protein from the muscle clearly reacted with a component in the hepatopancreas, brain tissues and plasma having the same molecular weight, demonstrated its wide distribution in several tissues. The finding of the accumulation of the protein in an acclimation temperature-dependant manner led us to the speculation that one of adaptive strategies to environmental temperature changes in temperate freshwater fish is *via* quantitative alterations of this protein.

Many studies concerning the effect of heat shock on cytosolic protein profiles have been performed (Lindquist 1986). HSP70 is one of the most intensively investigated HSPs and is known to be induced in organisms as diverse as human, invertebrate, and bacteria. In the case of fish, the complete structure of rainbow trout *hsp70*-related gene (*hsc70*) and its cDNA sequence have been reported (Zafarullah et al. 1992). The effect of acclimation temperature to the induction of *hsp70* in cultured cells of teleost has been reported for catfish and medaka (Koban, 1990).

The 65-kDa protein increasing in goldfish during warm temperature acclimation was similar to HSP70 proteins in terms of the isoelectric point, molecular weight and wide distribution in various tissues. However, relatively rapid changes in water temperature of the aquarium from 20 to 30°C during 20 h did not give rise to rapid increase of the 65-kDa protein. It took at least more than 2 days to increase the amount of this protein in goldfish muscle tissues. On the

contrary, HSP70 proteins are induced immediately, at least within 2 h after heat shock following an increase of temperature from 30 to 40°C in cultured cells of carp gill (Ku *et al.*, 1991).

Lindquist (1986) reviewed the complete amino acid sequences of HSP70 proteins from four eukaryotes and one prokaryote, suggesting that HSP70 is a highly conserved protein. If the 65-kDa protein is a member of HSP70 family, its N-terminal amino acid sequence may have considerable homology to that of HSP70 from human or those of other species. Figure 2-7 shows the N-terminal amino acid sequence of the goldfish 65-kDa protein in comparison with those of HSP70 proteins reported previously (Lindquist 1986; Zaharullah *et al.*, 1992). The sequence of the 65-kDa component was clearly different from those of HSP70 proteins from human, frog, rainbow trout, and *Drosophila*.

Although ATP-binding property has been claimed to be a fundamental property of the HSP70 proteins (Pelham 1986; Margulis *et al.*, 1989), the 65-kDa protein in muscle tissue extracts from the 30°C-acclimated goldfish did not bind to an ATP-agarose column. This was clearly demonsrated by immunoblot analysis. Whereas a 70-kDa component was absorbed to the ATP-agarose column and showed no reaction with the antibody raised against the 65-kDa component (see Fig. 2-8). These results indicated that the 65-kDa protein in goldfish does not belong to the family of so-called HSP70 proteins. Since the 70-kDa protein showing ATP-binding property from the 30°C-acclimated fish did not react with the antibody, This

protein is different from the 70-kDa component which appeared immediately after raising water temperature from 20 to 30°C (see Fig. 2-6). The functional and structural relationship of the 65-kDa protein with two types of the 70-kDa protein, remains to be elucidated.

The investigation in this chapter demonstrated the correlation of the 65-kDa protein with warm temperature acclimation of goldfish in terms of continuous responses of organisms to warm temperature. Thus this component was named warm temperature acclimation-related 65-kDa protein (Wap65) in goldfish. Although Wap65 may play an important role in gradual temperature acclimation process of goldfish, most characters of this protein were still masked.

Chapter 3

Isolation of goldfish Wap65

By immunoblot analysis with specific antibody, changes in levels of Wap65 in response to environmental temperature together with the ubiquitous nature of this component in goldfish were clearly demonstrated. Thus, this protein was considered to be one of the best candidates for monitoring the acclimation process at the protein synthetic level. Two dimensional electrophoresis could well separate Wap65 from others and supplied materials for developing specific antibody. However, a limited amount of the denatured protein due to the isolation method employed made it difficult to perform biochemical analysis including determination of the primary structure.

In this chapter, the warm temperature acclimation-related 65-kDa protein (Wap65) of goldfish was purified from epaxial muscle by a series of chromatographic steps while monitoring immuno-reaction with specific antibody. The N-terminal amino acid sequencing determined forty four residues and demonstrated that Wap65 was a new protein.

Materials and Methods

Materials

Goldfish (36 - 45 g) were acclimated to either 10 or 30°C for five weeks in laboratory aquariums as described in Chapter

1. After acclimation, fish from each temperature group were sacrificed and samples of white epaxial muscle were dissected out.

Column fillers used in high performance liquid chromatography (HPLC) and conventional chromatography included TSKgel DEAE-5PW, TSKgel G3000 SWG, TSKgel Phenyl-5PW and DEAE-Toyopearl 650M were purchased from Tosoh.

Standard proteins used to establish a calibration curve for molecular weight determination on gel filtration were purchased from Sigma and included blue dextran (2,000 kDa), β-amylase from sweet potato (200 kDa), alcohol dehydrogenase from yeast (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase from bovine erythrocytes (29 kDa), and cytochrome *c* from horse heart (12 kDa). Molecular weight markers for SDS-PAGE were also used as described in Chapter 1.

Preparation of muscle protein fraction

Muscle tissue extracts were prepared as described previously in Chapter 1. The resulting supernatants were used for electrophoretic analyses and $(NH_4)_2SO_4$ precipitation.

Isolation steps of Wap65

The fraction containing Wap65 was precipitated from the muscle extract at 50 - 80% saturation of $(NH_4)_2SO_4$. The precipitate was dissolved in and extensively dialyzed against buffer A containing 20 mM Tris-acetate (pH 7.5), 20 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and 0.05% NaN_3. The dialysate was then centrifuged at 33,000 x g for 20 min at

4°C. The supernatant containing the 65-kDa protein was applied to HPLC on a TSKgel DEAE-5PW column (0.75 x 7.5 cm) equilibrated with buffer A and proteins were eluted by a linear gradient of 20 - 500 mM NaCl. Fractions containing Wap65 were pooled and applied to high speed gel filtration using a TSKgel G3000 SWG column (2.15 x 60 cm) equilibrated with buffer B containing 50 mM potassium phosphate (pH 6.9), 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and 0.05% NaN_3. Fractions containing Wap65 were pooled and dialyzed against buffer C containing 25 mM Tris-HCl (pH 8.0), 7 M urea, 15 mM β-mercaptoethanol, and applied to a DEAE-Toyoperal column (1.6 x 8.0 cm) equilibrated with buffer C. A linear gradient with 300 ml of buffer C and 300 ml of buffer C plus 0.5 M KCl was used. The flow rate was set at 60 ml/h. Fractions containing Wap65 protein were exhaustively dialyzed against 0.05% DTT for 24 h and lyophilized. The sample was dissolved in 0.1% trifluoroacetate (TFA) containing 8 M urea and then applied to HPLC on a TSKgel Phenyl-5PW column (0.75 x 7.5 cm) equilibrated with 0.1% TFA. Absorbed proteins were eluted by a liner gradient of 0.7 - 70% acetonitrile.

Absorbance at 280 nm of effluents was monitored with a Shimadzu UV-160 spectrophotometer.

SDS-PAGE and immunoblott analysis were perforemed following each chromatographies.

Electrophoretic analyses

SDS-PAGE and two-dimensional electrophoretic analysis were performed by the method of Laemmeli (1970) and O'Farrell

(1975), respectively, as described in Chapter 1. Gels were stained with 0.1 % Coomassie Brilliant Blue R250 or silver nitrate (Oakley *et al.*, 1980) after electrophoresis as described in Chapter 1.

Immunoblot analysis

Immunoblot analysis was performed according to the method of Towbin *et al.* (1979) as described in Chapter 2.

N-Terminal amino acid sequencing

About 100 µg of the purified Wap65 was applied on the teflon seal of the cartridge block in the Applied Biosystems model 473A gas-phase sequencer.

Homology search

The protein homology search was performed by using the SWISS-PROT and National Biomedical Research Foundation (NBRF) 34 databases.

Results and Discussion

SDS-PAGE and immunoblot analysis

When proteins extracted from muscle were subjected to SDS-PAGE and subsequent immunoblot analysis, a strong reactivity was observed with Wap65 of the 30°C-acclimated goldfish (Fig. 3-1).

Ion-exchange HPLC

Most of Wap65 in the crude extract was precipitated by $(NH_4)_2SO_4$ ranging between 50 - 80% saturation. The precipitate was dissolved in buffer A and applied to a TSKgel DEAE-5PW column. The solution of the precipitate contained various components including a major component of about 45 kDa, creatine kinase (Nakagawa et al., 1988) (see Fig. 3-2A, lane 1). Washing with buffer A removed most proteins from the column. Wap65 was eluted at about 320 mM NaCl at the leading edge of the major peak (Fig. 3-2).

High speed gel filtration

Fractions containing Wap65 were pooled and applied to a gel filtration column of TSKgel G3000 SWG. As shown in Fig. 3-3, Wap65 was eluted after around fraction 19. High speed gel filtration with the extract gave a molecular weight of 65,000 for the immuno-reactive peak, suggesting that this protein is not composed of subunit structure.

Ion-exchange chromatography in the presence of urea

Considerable amounts of a 53-kDa protein were co-eluted with Wap65 from gel filtration column. To remove this impurity, the fractions containing Wap65 were dialyzed against buffer C containing 7 M urea and subjected to a DEAE-Toyopearl column equilibrated with the same buffer. SDS-PAGE of fraction 35 eluted at about 420 mM KCl gave a single band on silver nitrate staining. Wap65 was successfully separated from the 53-kDa protein on this column (Fig. 3-4).

Reversed-phase HPLC

To determine amino acid sequence, it is considered desirable to exclude any contamination as much as possible. Therefore, Wap65 separated on the DEAE-Toyopearl column was subjected to HPLC on a Phenyl-5PW column. The second peak eluted at 41% acetonitrile contained Wap65. This preparation gave a single band in SDS-PAGE (Fig. 3-5), which was subjected to analysis on a protein sequencer. The first peak possibly included an isoform of Wap65, since its N-terminal amino acid sequence was identical to that of the second peak as will be described in the following section. Other impurities in Figure 3-5 were successfully removed. The component in the second peak was subjected to two-dimensional electrophoresis, showing the same isoelectric point and molecular weight as those of Wap65 in the muscle extract (Fig. 3-6).

In this study, 517 μg of Wap65 was yielded from 23.4 g of epaxial muscle of the 30°C-acclimated goldfish.

N-Terminal amino acid sequence

The N-terminal amino acid sequence of Wap65 is shown in Figure 3-7. Though the 41st residue from the N-terminus could not be identified, totally 44 amino acid residues were determined.

Homology search by NBRF34 revealed that the 44 amino acid stretch of Wap65 was 29 and 26% homologous to spinach RNA polymerase β' subunit and human tyrosine hydroxylase, respectively (Fig. 3-7)(personal communication from T. Ooi, Kyoto Women's Univ.). Use of SWISS-PROT database also gave

similar results. RNA polymerase β' of 155 kDa is a subunit of RNA polymerase which is implicated in the transcription of spinach chloroplast (Hudson *et al.*, 1988). The relevant region is located in a C-terminal middle part of the β' subunit, whereas DNA binding site is in an N-terminal portion. Tyrosine hydroxylase of 50 kDa catalyzes formation of 3, 4-dihydroxyphenylalanine from tyrosine (Lodley *et al.*, 1985; Grima *et al.*, 1987). The homologous region is in the N-terminal domain which contributes to substrate specificity and regulation of enzyme activity, but has no catalytic activity. However, the significance of homologous regions of Wap65 remains uncertain. To identify Wap65, it is necessary to determine the primary structure of this protein. Determination of amino acid sequence in this chapter will facilitate such a course of investigation, cDNA cloning, which is described in Chapter 4.

Wap65 expressed during warm temperature acclimation was similar to heat shock proteins in terms of its increasing concentrations after temperature stress. Evidence has already been shown in Chapter 2 that Wap65 does not belong to the known family of HSP70. Further confirmation was obtained by homology search performed in this chapter, since the amino acid sequence of Wap65 was clearly different from those of any other HSPs.

It is requisite to perform biochemical and physiological experiments with chromatographically-purified Wap65 to elucidate its function at an individual level.

Chapter 4

cDNA cloning of goldfish Wap65 and its possible functions

In Chapter 3, Wap65 was isolated from muscle tissues of the 30°C-acclimated goldfish using a series of column chromatographies on TSKgel DEAE 5PW, TSKgel G3000 SWG TSKgel Phenyl 5PW and DEAE-Toyopearl 650M columns. Forty-four amino acids residues of Wap65 determined from the N-terminus showed an ambiguous homology with known proteins in database. However, these will be sufficient for developing polymerase chain reaction (PCR) primers.

In the present study, we isolated cDNA clones encoding Wap65 from the 30°C-acclimated goldfish to presume its physiological role and implication in temperature acclimation. It showed about 30% homology in the primary structure to mammalian hemopexins, but Wapp65 was significantly different from them in some presumed functional regions. In accodance with this result, plasma Wap65 was differnt from human hemopexin in terms of heme-binding properties. Transcription levels of Wap65 were clearly regulated by acclimation temperature.

Materials and Methods

Fish

Goldfish (15 - 24 g) were acclimated to either 10 or 30°C for five weeks in laboratory aquariums as described in Chapter

1. The acclimation period was determined in reference to the data in Chapter 2.

Blood sampling

Approximately 0.05 ml of blood was drawn from the caudal vasculature with a heparinized syringe fitted with a 23-gauge needle after anesthetizing fish with 600 ppm of 2-phenoxyethanol (Wako). For heme-affinity chromatography, 0.5 ml of plasma was taken from the 30°C-acclimated goldfish. Blood samples were centrifuged at 3,000 rpm, and plasma was stored at -20°C until electrophoretic analysis. Precipitated hemocytes were subjected to RNA isolation.

RNA preparation and cDNA synthesis

Total RNA was extracted from various tissues of temperature-acclimated goldfish according to the guanidium isothiocyanate procedure (Sambrook *et al.*, 1989) or the manufacturer's protocol with RNA extraction solution (Isogen, Nippon Gene). Poly(A) RNAs were isolated with oligo(dT)-cellulose spin columns (Takara) and their corresponding cDNAs were synthesized using Amersham cDNA synthesis kits.

Polymerase chain reaction conditions

The conditions for PCR were as follows. Ten µg each of 5' and 3' primers and 100 ng of a given template DNA were combined with 10 µl of 10 × Tth DNA polymerase buffer (67 mM Tris-HCl, pH 8.8,16.6 mM $(NH)_4SO_4$, 6.7 mM $MgCl_2$ and 10 mM 2-mercaptoethanol) and 2 µl of 20 mM dNTP solution. The volume

was brought to 100 µl with H_2O, and the mixture was overlaid with 50 µl of mineral oil to prevent evaporation. Two units of Tth DNA polymerase was added to the reaction mixture, and the cycle reaction was initiated. Denaturation was at 94°C for 1 min, annealing at 55 ~ 65°C for 2 min, and polymerization at 72°C for 1 min. The cycle was repeated 30 times.

Construction and screening of a cDNA library

A hepatopancreas cDNA library was constructed in lambda ZAP II vectors (Stratagene) using cDNAs prepared from the hepatopancreas of the 30°C-acclimated goldfish. cDNAs synthesized were blunt-ended with T4 DNA polymerase, tailed with *Eco*RI plus *Not*I adapters (GIBCO BRL), size-fractionated in agarose gel, and ligated into the *Eco*RI sites of the ZAP II vector. Following packaging and amplification, the resultant library was screened employing the plaque hybridization method with randomly labeled [^{32}P] DNA probes. Positives were plaque purified and the inserts were excised in the form of pBluescript SK plasmid vectors according to the manufacturer's protocol. The plasmid DNAs were purified utilizing an alkaline lysis method (Sambrook *et al.* 1989) and used for further analysis.

Sequencing analysis

Sequencing was performed for both strands on subclones deleted by exonuclease III and mung bean nuclease (Barnes *et al.*, 1983), with a DNA sequencer model 373A using dye deoxy

terminator cycle sequencing kits (Applied Biosystems). The protein homology search was performed by using the SWISS-PROT database coordinated with the Inherit program (Applied Biosystems).

Northern blot analysis

Ten µg of total RNA isolated from the goldfish tissues were denatured at 65°C for 15 min in 50% formamide, and subjected to electrophoresis on a 0.7% agarose gel in 0.2 M 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.0, containing 2.2 M formamide, 0.05 M sodium acetate and 5 mM EDTA, then transferred to Hybond N$^+$ nitrocellulose membranes (Amersham). Total hepatopancreatic RNA, primarily composed of 18 and 28 S rRNAs, was treated in the same manner as above and used as size markers. The membranes were air-dried and baked at 80°C for 15 min prior to hybridization with randomly labeled [^{32}P] DNA probes. Membrane filters were washed at 65°C with several buffer changes of decreasing SSC concentrations from 5× to 0.1× and autoradiographed on X ray films with intensifying screens at -80°C. The hybridized membranes were scanned by a Fujix BAS 1000 computerized densitometer scanner and quantified using a recommended scanning program.

Isolation of genomic DNA and Southern blot analysis

Genomic DNAs were isolated by homogenizing the male goldfish hepatopancreas and subsequently treated with proteinase K (Gross-Bellard *et al.*, 1972). For Southern blot analysis, 20 µg of genomic DNAs were digested with a series of

restriction endonucleases, and the digests were electrophoresed in 0.7 % agarose gels. The gels were processed with slight modifications after Sambrook *et al.*(1989), denatured with 0.5 M NaOH containing 1.5 M NaCl, transferred to nylon membranes omitting renaturing steps, and baked at 80°C for 15 min. The 5' DNA fragment of pw65-1 encoded Wap65 was obtained by digestion with *Eco*RI, and used as a probe. Membranes were hybridized with randomly labeled [^{32}P] DNA probes and washed under stringent conditions in the Northern blots.

cDNA cloning of goldfish β-actin

cDNAs coding for β-actin were isolated from a goldfish hepatopancreas cDNA library with a DNA probe coding for β-actin of medaka, *Oryzias latipes*, provided by Dr. Takashi Aoki, Tokyo University of Fisheries.

Electrophoretic analysis

SDS-PAGE and two dimensional electrophoresis were performed by the method of Laemmli (1970) and O'Farrell (1975), respectively, as described in Chapter 1. Gels were stained with 0.1% Coomassie Brilliant Blue R250 after electrophoresis. The sample volume used was 3 µl of plasma for analysis. Protein concentrations were measured by a Simadzu CS-9300PC densitometer using a recommended program referring to the standard curve of bovine serum albumin (Sigma).

N-terminal amino acid sequencing

The N-terminal amino acid sequence was determined by the method of Matsudaira (1987) as described in Chapter 2.

Heme affinity chromatography

Heme affinity chromatography was performed according to Tsutsui and Muller (1982). A portion of 0.5 ml of plasma obtained from the 30°C-acclimated fish or human was dialyzed overnight against 10 mM sodium phosphate (pH 7.5) containing 100 mM NaCl. Before dialysis, 25 µg aprotinin/ml and 10^{-9} M PMSF have been added to plasma. Samples were then mixed with 1 ml of heme agarose (Sigma) and mixtures were placed in darkness overnight at 4°C. The slurry was filtered through columns and washed with 30 ml of 10 mM sodium phosphate (pH 7.5) containing 0.5 M NaCl and 10^{-9} M PMSF. After elution from the heme agarose with 9 ml of 0.2 M sodium citrate (pH 5.2) containing 50 mM NaCl and 0.02% NaN_3, the eluted proteins were concentrated on an Ultrafree C3TK (Millipore) and analyzed by SDS-PAGE and subsequent immunoblot analysis. The heme-agarose were treated with the same volume of the SDS-PAGE sample buffer described in Chapter 1. After incubation at 100°C for 3 min, 10 µl of the mixture was loaded into SDS-PAGE gel.

Statistical analysis

The Student's t-test was used for statistical analysis.

Results

PCR amplification of a DNA fragment encoding an N-terminal region of Wap65

Forty-four amino acids of Wap65, determined by N-terminal amino acid analysis in Chapter 3, were sufficient for developing PCR primers (Fig. 4-1). The author synthesized three sets of oligonucleotides encoding three peptides of Wap65 (Fig. 4-1). Either a *Bam*HI or *Eco*RI linker was added to each primer to facilitate subsequent analysis.

The author used cDNAs synthesized from poly(A) RNA preparations of the 30°C-acclimated goldfish muscle as templates. The first PCR was carried out at an annealing temperature of 55°C with primers 1 and 2, yielding many products of 50 ~ 300 bp due to nonspecific amplification. PCR products with sizes from 80 to 150 bp were eluted from agarose gels. The second PCR was performed with primers 1 and 3 using the eluted PCR products as templates, yielding a DNA fragment of 102 bp which was consistent with the size expected from the amino acid sequence between the two primers. Subsequently, the 102 bp product was blunted and digested with *Bam*HI and *Eco*RI, then subcloned into pUC118 vectors. The amino acid sequence deduced from a clone pw65-N, concurred with that directly determined for the isolated protein, GANLDRCGGMEFDAIAV.

Northern blots with the pw65-N clone were performed to examine transcription levels of Wap65 mRNA in several tissues including hemocytes, hepatopancreas, muscle and brain. The

highest amount of Wap mRNA was observed in the 30°C-acclimated hepatopancreas among tissues examined. Unexpectedly, mRNA levels were markedly low in the muscle which was used for isolation of Wap65 and subsequent analysis of the N-terminal amino acid sequence (Fig. 4-2).

cDNA cloning of Wap65

In order to isolate the clones that cover the entire coding region of Wap65, the author constructed a new cDNA library from the 30°C-acclimated goldfish hepatopancreas. Screening of 1.0×10^5 plaques probed with the aforementioned pw65-N DNA fragment of 102 bp yielded four clones, pw65-1 ~ 4. The longest clone, pw65-1, had 1749 bp with initiator and terminator codons. It contained two *Eco*RI sites and one each of the *Pst*I and *Bam*HI sites. These restriction endonuclease sites, together with a series of ad hoc deletion mutants, facilitated determination of the DNA nucleotide sequence. However, no clones containing a putative polyadenylation site were found (Fig. 4-3). In order to obtain such sites, the library was rescreened with pw65-1, yielding additional five positive clones. The longest clone, pw65-5, from the second screening contained one polyadenylation signal together with a poly(A) tail, but lacked a part of 5' coding region (Fig. 4-3).

DNA nucleotide and deduced amino acid sequences from the two clones are shown in Figure 4-4. In total, 1761 bases were determined where 1335 bases encoded 445 amino acid residues. The coding region was followed by a 3' noncoding region of 341

bp that contained a polyadenylation signal, AATAAA, in 17 bp upstream from the poly(A) tail. The first methionine was followed by a short polypeptide rich in hydrophobic amino acids that may serve as a signal peptide for secreting Wap65 across cell membranes. This peptide, consisting of 30 amino acids, was followed by aspartic acid, which was identified as the N-terminal amino acid of Wap65 isolated from the warm-acclimated goldfish muscle in Chapter 3.

The molecular mass from deduced amino acids was 47.5 kDa, which is smaller than that determined by SDS-PAGE (65 kDa). Three possible glycosylation sites of Wap65 may explain these differences (Fig. 4-4).

Homology search for protein sequence

A homology search for the Wap65 deduced amino acid sequence was conducted using the SWISS-PROT database, revealing that some parts of Wap65 contained the sequences similar to those of hemopexin from rat and human (Takahashi *et al.*, 1985; Nikkila *et al.*, 1991). The overall homology of goldfish Wap65 to rat hemopexin was 31%. Hemopexin is a serum glycoprotein that is mainly synthesized in the liver, and plays an important role in scavenging hemes from the blood. It is highly conserved in its primary structure and shows 78 % homology between human and rat (Nikkila *et al.*, 1991). Ten out of twelve cysteine residues in hemopexin were conserved in Wap65, indicating that the disulfide bridges may be similarly arranged in the two proteins (Fig. 4-5) (Takahashi *et al.* 1985). A comparison of the N- and C-terminal halves of

goldfish Wap65 resulted in 22% homology, suggesting that an internal duplication event had occurred in goldfish as has been described for human and rat hemopexins (Altrude *et al.*, 1985, Nikkila *et al.*, 1991). Seven of eight internal repeats characteristic of the "pexin" gene family reported by Jenne and Stanley (1987) were also observed in Wap65 (Fig. 4-5).

Despite such similarity, there were significant differences between Wap65 and mammalian hemopexins. Wap65 contained no tryptophan, which is unusually abundant in mammalian hemopexins (Fig. 4-5) (Muller-Eberhard and Liem, 1974). Furthermore, Wap65 had few of the possible glycosylation sites which seem to involve heme binding in human hemopexin (Sato *et al.*, 1994) (Fig. 4-5).

Genomic organization

To analyze the genomic organizations of Wap65, Southern blot experiments were carried out probing with an *Eco*RI restriction fragment of the pw65-1 clone harboring 217 bases from the 5' terminus. Probing hepatopancreas genomic DNA with the above DNA fragment gave one band each of 2.1, 5.4 and 2.0 kbp after digestion with *Eco*RI, *Bam*HI and *Pst*I, respectively (Fig. 4-6). These results suggest that Wap65 is encoded by a single copy gene. The occurrence of two bands observed after *Hind*III digestion suggests a possible presence of an intron with a *Hind*III site inserted into the corresponding region to the probe, since no *Hind*III site was found in the Wap65 cDNA.

Effects of acclimation temperature on Wap65 transcription

levels

The pw65-1 DNA clone was used as a probe for investigating changes in mRNA levels of Wap65 in the hepatopancreas after 30 and 10°C acclimation. As seen in Fig. 4-7A, a single transcript was observed by RNA blot analysis. Levels of these hybridized transcripts were determined with a BAS 1000 densitometer, revealing that temperature acclimation of goldfish from 10 to 30°C resulted in a 10-fold increase at the mRNA level of Wap65 without appreciable changes in molecular weight. These changes were significant at $P < 0.01$, even after transcriptional levels of Wap65 were standardized with those of β-actin (Fig. 4-7B).

The occurrence of Wap65 in the serum

The tissue-specific transcription of Wap65 revealed by the Northern analysis in this study, together with a nonspecific distribution of the translated product shown in Chapter 2, led us to postulate that Wap65 may be circulated as a plasma protein after being synthesized in the hepatocytes. Two-dimensional electrophoretic analysis in Chapter 1 had demonstrated that the 30°C-acclimated goldfish showed an increased abundance of the 65-kDa protein (Fig. 1-8), possibly corresponding to Wap65 in muscle tissue extracts. The N-terminal amino analysis following two-dimensional electrophoresis (Fig. 4-8) resulted in DEPQGHQ. These results confirmed that this plasma protein was identical to Wap65 from muscle extracts. A diffused band observed in the acrylamide gel may be due to multiple glycosylation levels of this

protein as expected from its amino acid sequence. Plasma levels of Wap65 in the 30°C- and 10°C-acclimated fish were determined at 1.93 ± 0.20 and 0.22 ± 0.02 mg/ml, respectively, with a Simadzu CS-9300PC densitometer. Temperature acclimation of goldfish from 10 to 30°C resulted in a 10-fold increase of Wap65 as in the case of its transcripts. These changes were significant at $P < 0.01$.

Heme affinity chromatograph

To examine a heme binding property of Wap65 implied by a significant homology with hemopexin, an established method to isolate mammalian hemopexin was adopted. Figure 4-6A shows results of SDS-PAGE analysis on heme affinity chromatography of the plasma obtained from the 30°C-acclimated goldfish and human. An upward arrow on immunoblott membrane (Fig. 4-9B) indicates Wap65 removed from the column by washing with 10 mM sodium phosphate buffer (pH 7.5) containing 0.5 M NaCl. In contrast, a 60-kDa component of human plasma absorbed to the column was eluted with 0.2 M sodium acetate buffer (pH 5.2) (Fig. 4-9A). This protein appeared to be human hemopexin in terms of molecular weights and heme binding property (Tsutsui and Mueller, 1982). Identification of three plasma components of goldfish showing affinity for the column remained obscure (Fig. 4-9A).

Discussion

The purpose of the study described in this chapter was to clone cDNAs encoding the warm temperature acclimation-related protein, Wap65, and to examine its possible functions which may be defined by its DNA nucleotide sequence.

A homology search with protein databases revealed that Wap65 had overall 31% homology to and shared several homologous regions with rat hemopexin. Hemopexin is a mammalian serum glycoprotein that transports heme to liver parenchymal cells *via* a receptor-mediated process (Muller-Eberhard and Liem, 1974). A high conservation of cysteine residues was seen between Wap65 and mammalian hemopexin, suggesting similar disulfide bridges in the two proteins (see Fig. 4-5) (Nikkila *et al.*, 1991). Several other regions showed very high homology between Wap65 and mammalian hemopexin, implying that Wap65 may have physiological functions similar to those of hemopexin. It seems that mammalian hemopexin and Wap65 belong to the same gene family. Despite of the above similarities, distinct structural differences between Wap65 and hemopexin aroused the question whether they can share the same function. A 31% homology at the amino acid level between the two proteins was not so high as a 78% homology between rat and human hemopexins. Furthermore, no tryptophan was found in Wap65. It has been reported that the content of tryptophan is unusually high in hemopexins and several heme binding proteins, suggesting that certain tryptophan residues are essential for their

interaction with heme (Muller-Eberhard and Liem 1974).

Satoh *et al.* (1994) expressed a recombinant human hemopexin in baculovirus-infected insect cells with site-directed mutagenesis and demonstrated that N-linked glycosylation and His-127 are essential to bestow a high affinity of hemopexin for blood circulating hemes. Wap65 contained no histidine residue in the region of His-126 in rat or His-127 in human hemopexin, and was poor in possible glycosylation sites as compared with rat and human hemopexins (Takahashi *et al.*, 1984) (see Fig. 4-5). Therefore, Wap65 is not expected to have the same heme binding properties as conventional hemopexins, which was further confirmed by the result of heme affinity chromatography (see Fig. 4-6). Alternatively, histidine residues in Wap65 at the sites different from those of mammalian hemopexins may serve as heme axial ligands, causing interaction of Wap65 with heme in a different manner.

Northern blot analysis suggested that the increased abundance of Wap65 translation levels in the hepatopancreas in response to warm environmental temperature shown in Chapter 1 appears to be regulated for the most part by its increased mRNA levels. Though the presence of Wap65 mRNA in muscle tissue was demonstrated in PCR experiments, Wap65 in plasma as well as in muscle and brain shown in Chapter 2 seems to be transported from the hepatopancreas as in the cases mammalian hemopexins (Kushner, 1988). It is well established that the concentration of a subset of acute phase proteins (*e. g.* α_1-acid glycoprotein, α_1-antitrypsin, ceruloplasmin, C-reactive

protein, fibrinogen and haptoglobin) is increased in a pathological state following infection in mammals (Koj, 1974). These acute proteins include hemopexin and their levels are considered to be mediated under cytokine regulations during inflammation (Poli and Cortes, 1989). Two dimensional electrophoretic analysis accounts for our tentative conclusion that no possible goldfish acute phase proteins except for Wap65 increase their quantities in the blood during warm acclimation (see Fig. 4-8). Thus the regulation of Wap65 expression under warm temperature acclimation may be different from that of mammalian hemopexins during inflammation. Though an upstream regulation in the gene expression of Wap65 is still ambiguous, such regulatory systems for temperature acclimation may exist widely in poikilotherms whose body temperatures are closely related to those of environment.

Chapter 5

Structure and expression of goldfish Wap65 gene, *wap65*

In Chapter 4, Northern blot analysis demonstrated that levels of Wap65 mRNA in hepatopancreas were higher than those in hemocytes, muscle and brain, suggesting that the gene encoding Wap65, *wap65*, is transcribed specifically in hepatopancreas. In addition, comparison for Wap65 mRNA levels in hepatopancreas of the 30°C-acclimated fish with those of the 10°C-acclimated one was performed in Chapter 4, revealing that the abundancy of this transcript was markedly increased as a result of warm temperature acclimation. Few investigations have focused on temperature adaptation of fish at the gene expression level except for antifreeze protein in winter flounder (Chan *et al.*, 1993). However, Vaisius *et al.* (1989) demonstrated that gene transcription of antifreeze protein was not responsive to temperature. In contrast, Wap65 seems to be a promising protein for investigating metabolic regulation by ambient temperature in teleost at the transcriptional level.

In this chapter, firstly, tissue-specific expression of *wap65* was ascertained by the reverse transcription-PCR method (RT-PCR). Secondly, changes in Wap65 mRNA levels were analyzed during the temperature acclimation process. In addition, a 5' region of *wap65* was cloned to understand the molecular mechanisms involved in the regulation of its expression. In this experiment, sequencing of a presumed promoter region of *wap65* revealed the presence of motifs

similar to cytokine responsive elements, suggesting that *wap65* may also be involved in responses to inflammation. In mammals, systematic injury enhances the hepatic production of several plasma proteins namely the acute phase reactants including hemopexin (Koj, 1974). Cytokine responsive motifs have been described for several acute phase protein genes (Poli and Cortes, 1989). To examine such possibility, lipopolysaccharide (LPS) administrations into goldfish were carried out.

Materials and Methods

Fish

Goldfish (15 - 24 g) were acclimated to either 10 or 30°C for five weeks in laboratory aquariums as described in Chapter 1. After acclimation period, the 30°C-acclimated fish (19 g) was used for RT-PCR experiment to examine tissue-specific expression of *wap65*.

To examine the effect of raising water temperature on the levels of Wap65 mRNA, 20 individuals from the 10°C-acclimated group were treated with increased temperatures to 30°C in an aquarium of 60 x 40 x 30 cm equipped with a thermocontroller (Toho, TP-173) over a 20 h period. After 4 h resting at 30°C, 4 fish were taken out from the aquarium to analyze levels of Wap65 transcripts in hepatopancreas. Other groups each containing 4 sample fish were taken out periodically from the 30°C-aquarium over a 22 day duration (see Fig. 5-2). In the

case of lowering water temperature from 30 to 10°C, the corresponding sampling procedure was followed (see Fig. 5-3).

The 10°C-acclimated fish were also subjected to Northern blot analysis following LPS administration.

RNA Preparation

Total RNA was obtained according to the manufacturer's protocol as described in Chapter 4.

Reverse Transcription Polymerase Chain Reaction Conditions

First-strand cDNA synthesis was performed with Ready-To-Go™ T-Primed First-Strand kit (Pharmasia) using 5.0 µg of total RNA from hepatopancreas, intestine, ovary, heart, gill, eye, skin, hemocytes, muscle, brain of the 30°C-acclimated goldfish. For PCR reaction, a pair of primers were synthesized; the 5' primer (5'-TTGGATGCTGCAGTGGAGTGTC-3') and the 3' primer (5'-TGCACACACAAAGGCAGCATCT-3') are located at 484 ~ 505 bp and 1086 ~ 1107, respectively, downstream from the first nucleotide of Wap65 cDNA clone, pw65-1, shown in Fig. 4-3 in Chapter 4. PCR was carried out according to instructions provided with the kit. Briefly, 5 µg of the reverse transcription reaction mixture from each sample was added into a final volume of 20 µl containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 0.65 mM of each dNTP, a set of 5' and 3' primers (0.5 mM) and 1 unit of Taq DNA polymerase (Takara). The cycle reaction was repeated 30 times with denaturation at 94°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 1 min. After PCR,

the amplified DNA products were separated on 2% agarose gel and visualized by staining with ethidium bromide. For confirmation of the first-strand synthesis, PCR amplification of β-actin was carried out. The 5' primer (5'-ATGTTCGAGACCTTCAACACC-3') and the 3' primer (5'-TCTCCTGCTCGAAGTCAAGA-3') were synthesized, based on the known cDNA coding for β-actin of goldfish described in Chapter 4.

Northern blot analysis

Northern blot analysis was performed as described in Chapter 4. The quantified mRNA levels of Wap65 were statistically analyzed using the Student's t-test.

Isolation of genomic DNA

Genomic DNAs were isolated as described in Chapter 4. After digestion with *Eco*RI, 20 μg of genomic DNA was subjected to electrophoresis for size-fractionation.

Construction and screening of a genomic DNA library

Genomic DNA fragments of about 2.0 kbp digested with *Eco*RI were obtained by size-fractionation in an agarose gel, and ligated into the *Eco*RI sites of λZAP II vectors (Stratagene). Following packaging and amplification, the resultant library was screened as described in Chapter 4.

Sequencing analysis

Sequencing was performed as described in Chapter 4.

Primer extension analysis

A 22 bp oligonucleotide (5'-GCAGCGAGACTCAATGAGAGAG-3') complementary to the sequence of 62 - 83 bp downstream from the first nucleotide of Wap65 cDNA clone, pw65-1, (see Chapter 4) was synthesized, end-labeled with T4 polynucleotide kinase, and used as a primer for the reverse transcriptase reaction according to the published procedures with slight modifications (Sambrook *et al.*, 1989). Briefly, 30 µg of total RNAs were hybridized for 15 h with the end-labeled oligonucleotide in 40 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) (pH 6.4) containing 1 mM EDTA, 0.4 M NaCl and 80% formamide. Reverse transcriptase, Superscript™II (GIBCO BRL), was used to extend this primer to produce cDNA complementary to the RNA template. The length of the extension products was measured by electrophoresis through a 6% polyacrylamide sequencing gel containing 6 M urea. The genomic clone, pGE-1, encoding Wap65 was sequenced by dideoxy method using *Bca*best sequencing kit (Takara) and also subjected to the same electrophoresis for comparison.

LPS administration into goldfish

A solution of 100 µl saline containing 500 µg of LPS from *E. coli* (Sigma) was injected *i.p.* Control fish received saline only. On day 4 after the treatment, fish were killed and RNA was extracted from the hepatopancreas. Sampling schedule was determined referring to preliminary works together with a study on fish inflammatory reactions reported by Anderson *et al.* (1975).

Results

Tissue-specific expression of wap65

RT-PCR for hepatopancreas, intestine, ovary, heart, gill, eye, skin, hemocytes, muscle, brain was carried out to overcome the an insufficient sensitivity of Northern analysis. PCR amplification of Wap65 cDNA with the primers was expected to produce a fragment of 623 bp. As shown in Fig. 5-1 amplification of reverse-transcribed RNA from hepatopancreas, heart and muscle gave the products of an expected size. In the case of brain, many bands were observed, indicating an inaccurate amplification of Wap65 mRNA with the set of primers. Amplified products of β-actin with the size of 314 bp ensured the success of first strand synthesis in all tissues.

Changes of Wap65 mRNA levels in hepatopancreas of goldfish in the process of temperature acclimation.

In accordance with raising water temperature shown in Fig. 5-2, a marked accumulation of Wap65 mRNA was clearly demonstrated by Northern analysis. Because changes in hepatopancreatic Wap65 mRNA contents following temperature shifts were associated with no apparent changes in transcript size, Wap65 mRNA levels were quantified using a Fujix BAS 1000 densitometer after Northern blot analysis. As shown in Fig. 5-4A, the abundance of hepatopancreatic Wap65 mRNA increased 20-fold within 4 h after raising water temperature to 30°C and

this increase reached a maximum of 40-fold on day 3 after the shift of water temperature. Then the levels of Wap65 mRNA tended to decrease and reached 10-fold on day 21 after raising water temperature compared with the levels of 10°C-acclimated fish. This concentration on day 21 at 30°C was assumed to be the steady state levels in the 30°C-acclimated goldfish, considering the abundance of Wap65 mRNA in goldfish acclimated to 30°C for 5 weeks (see Fig. 5-4B, day -1). All these changes for the levels of Wap65 mRNA compared with the level in initial sample fish were significant at $P < 0.01$. In the case of lowering water temperature according to the time schedules shown in Fig. 5-3, Wap65 mRNA levels were decreased by 50% after the temperature shift over 20 h and continuously decreased, further reaching 1/10-fold on day 21 comparing to the levels of Wap65 mRNA in hepatopancreas from 30°C-acclimated goldfish (Fig. 5-4B, day 21 and 5-4A, day -1). Differences in the level of Wap65 mRNA between fish groups on day -1 and 21 from that of the 30°C-acclimated fish was significant at $P < 0.05$. In contrast, effects of temperature sifts on the levels of β-actin mRNA in hepatopancreas were not apparent (Figs. 5-4C and D).

Genomic DNA cloning of wap65 containing a 5'-flanking region and its sequencing

Southern blot analysis in Chapter 4 indicated that there was only one copy of Wap65-coding gene, *wap65*, in the goldfish genome and that a 2.1 kbp genomic DNA fragment after digestion with *Eco*RI contained exon encoding a region proximal to N-

terminus of Wap65. To isolate a 5'-flanking region of *wap65*, genomic library was constructed and sequenced. Screening of 2 X 10^5 plaques probing with the *EcoRI* fragment of the pw65-1 clone described in Chapter 4 gave three clones, pGE-1 ~ 3. Since the sequencing analysis of 5' and 3' ends of these clones together with the restriction mapping suggested that all three clones were identical, only pGE-1 was subjected to further analysis. The clone was formed to have the sequence ranging from -369 in 5'-flanking region to +1465 in coding region of Wap65. Sequence comparison of the genomic clone, pGE-1, with the cDNA clone, pw65-1 revealed the presence of four introns in pGE-1. All donor and accepter junctions for splicing were in the GT/AG rule (Breathnach et al., 1978). The organization of pGE-1 encompassing 5'-flanking region and four introns of *wap65* is shown in Fig. 5-5.

Determination of transcription start point

Primer extension analysis was carried out to identify transcription start point, giving one band for the 30°C-acclimated fish (Fig. 5-6, lane 1). No other transcription sites were obtained within 150 bp upstream and 100 bp downstream of this site. Therefore, the adenine located 46 bases upstream from the putative translation start site was suggested to be as a transcription start point for 30°C-acclimated fish. Unexpectedly, goldfish killed 3 days after raising water temperature from 10 to 30°C gave different transcription start point, the thymine located one base upstream from aforementioned adenine (Fig. 5-6, lane 2).

Identification of Potential transcriptional regulatory element in a 5' region of wap65

A search for putative transcriptioinal elements in the 1.8-kbp *Eco*RI fragment encompassing the 5' region of *wap65* was carried out by use of SIGNALSCAN (Ver 3.3, 1993; Prestridge, 1991). This revealed a TATA box located at -28 and a reversed CAAT box located at -247. The following transcription factors reported in vertebrates were also found in the upstream of transcription initiation points and in introns: NF-IL6 (-252, -90, 348, 674), HNF-1 (699), AP-1 (-283), GATA-1 (-345, -307, 853), TFIID (-144, 658, 813), v-Myb (-332, -247), NFκβ (446), XREb (-59), MRE (-159), overlapped repeat of GH1 (1027 ~ 1043). These sites are indicated in Fig. 5-7. References for transcription factors in vertebrate were compiled by Faisst and Meyor (1992).

Comparison of the 5' upstream sequence of wap65 with that of human hemopexin gene

There was no apparent homology in the putative promoter sequences between *wap65* and human hemopexin gene except for NF-IL6 and an IL6 responsive element (IL-6-RE) (Fig. 5-8). These elements in mammals were characterized to bind to the nuclear factors which are induced by interleukin-6 in hepatocyte and act as enhancers for hemopexin transcription in inflammatory condition (Immenschuh *et al.*, 1994).

Effect of LPS injection into goldfish on Wap65 transcripts levels

Since analysis of the 5' upstream region of *wap65* indicated the possibility that *wap65* transcription might be activated by mediators in immune system as in the case of rat hemopexin, the 10°C-acclimated goldfish were given i. p. injection of LPS. As seen in Fig. 5-9, Northern blot analysis revealed that LPS injection in goldfish resulted in a 2-fold increase in mRNA levels of Wap65 in hepatopancreas without significant changes in mRNA levels of β-actin. Although functional *cis*-elements responsible for this reaction remain to be elucidated, these results suggest the involvement of Wap65 in immunological response in goldfish. For further information, time-dependent changes in Wap65 mRNA levels under the infected conditions together with those in deletion mutants under the reporter assay system should be examined.

Discussion

Since the amount of PCR products reaches plateaus when necessary components in PCR are consumed, it can be difficult to obtain a quantitative information exactly on the products amplified (Alice et al., 1989). However, relative and rough comparisons of mRNA levels seems possible before the PCR products reach the plateau. As shown in Fig. 5-1, the result obtained by RT-PCR obviously demonstrated that the hepatopancreas contains the highest levels of Wap65 mRNA among tissues examined for goldfish. Since the levels of Wap65 mRNA in muscle was extremely low compared with those in

hepatopancreas in spite of an exponential nature in amplification by PCR, it was plausible to consider that the most parts of Wap65 observed in the soluble protein fraction of muscle was transported from hepatopancreas through the circulation system. Hepatopancreas-specific expression of *wap65* led us to postulate that the adaptation of fish to fluctuated environmental temperature may be by tissue-specific functions. Saez *et al.* (1984) reported that the hepatocytes from winter carp undergo significant changes in its cytoarchitecture different from that of summer fish. Together with a liver-specific synthesis of antifreeze proteins in marine teleost, it is possible that fish liver might play important roles in adaptation to fluctuated temperatures at an individual level.

Kinetic analysis of Wap65 mRNA following temperature shifts demonstrated a direct correlation of Wap65 transcript levels with ambient temperature shifts. In the case of raising water temperature, the levels of Wap65 were regulated in a complexed manner (Figs. 5-2 and 5-4). Levels of Wap65 transcripts remained high within 9 days after raising water temperature. Thereafter, goldfish showed the constant transcript levels which were equivalent to those found in fish acclimated to 30°C for five weeks (Fig. 5-4B, day -1). It was plausible to consider that such regulation may be issued by a feed back control system. To disclose such regulatory mechanism, run-on-assay for *de novo* synthesis of Wap65 transcripts is required.

Two different transcription start points revealed by

primer extension analysis seems to give further confirmation of the complexed regulation of *wap65* expression in response to raising water temperature. Alternatively, such differences could be caused by individual variation due to the point mutation of genom.

Although many putative transcription elements were found in the 5' region of *wap65*, most sites are probably not functional in the control of *wap65*. However, NF-IL6 and HNF-1 which bind to liver-specific transcription factors have high possibility to be functional, since *wap65* is highly and specifically expressed in hepatopancreas. Some of these elements were located at introns in goldfish *wap65*. In human, tissue-specific transcriptional enhancers of the apolipoprotein B gene have been reported to be in the second intron (Brooks and Levy-Wilson, 1992), and their functions were demonstrated by deletion experiments. In order to reveal the *cis*-element responsible for enhancing the transcription of *wap65*, systematic deletion analysis for 5' upstream and intron regions should be performed using primary culture of goldfish hepatopancreas as a transient expression system.

The 5' flanking region of *wap65* contained sequences similar to enhancer motifs of NF-IL6 and IL-6-RE in human. Though interleukin-6 has not been identified in fish, it is highly possible that some triggers for enhancing *wap65* transcription are a family of cytokines which induce nuclear factors interactive with the motifs similar to human NF-IL6 and IL-6-RE.

To verify whether Wap65 transcription is enhanced by

mediators in immune system, the 10°C-acclimated fish were received *i. p.* injection of LPS. After LPS administration into goldfish, levels of Wap65 transcripts was increased similarly as in response to warm temperature. These results suggested that the gene expression of Wap65 is moulated by ambient temperature together with immunological stimulation.

Several reports have shown that infected ectotherms prefer a relatively hot area, resulting in acquisition of beneficial effects to overcome infections (Kluger *et al.*, 1975; Reynolds *et al.*, 1976; Covert and Reynolds, 1977). For example, goldfish infected with bacteria selected their environment at 32°C and therefore had higher survival rate compared with fish whose body temperature was maintained at 25°C (Covert and Reynolds, 1977). Although detailed aspects of the mechanisms involved in the increase in self-defense ability from bacterial infection in fish with increasing body temperature are unknown, there may be a synergism between response to warm temperature and self-defense from infections. The gene expression of Wap65 also seems to involved in some common mechanisms for warm temperature acclimation and self-defence.

It has been reported for fish that several components of the self-defense mechanisms including endocytosis of macrophage (Pxytycz and Jozkowicz, 1995) and mitogen-induced proliferation of leukocyte (Le-Morvan *et al.* 1995) are temperature-dependant. The remarkable accumulation of Wap65 mRNA in goldfish hepatopancreas was observed in response to raising water temperature without apparent bacterial infection

(Fig. 5-2), implying that the response of Wap65 could not be fully attributed to a temperature-dependent nature of immune response. Hence, it is speculated that Wap65 is functional in both responses to increased temperatures and infection. Though physiological roles of Wap65 are still unknown, it is expected that understanding regulatory function of Wap65 will provide new insights in the relevance of acclimatory systems to immune systems.

General Discussion

In this study, the warm temperature acclimation-associated 65-kDa protein, Wap65, was found and characterized, revealing that temperature acclimation of goldfish involves the control in gene expression of this protein at the transcript level. Wide distribution of Wap65 by blood circulation in response to warm water temperature suggests the involvement of this protein in warm temperature acclimation. The deduced amino acid sequence of Wap65 was 31% homologous to rat hemopexin. However, Wap65 was significantly different from mammalian hemopexins in functional regions, implying its possibly different functions. Since *wap65* was specifically expressed in the hatopancreas of goldfish, liver-specific enhancer motifs found in the 5' region of *wap65* are highly possible to be functional. The abundancy of Wap65 mRNA markedly increased in response to warm water temperature. Plasma Wap65 levels showed a good correlation to those of the corresponding transcript in the hepatopancreas, suggesting that plasma Wap65 concentrations are regulated mainly by transcript levels in the hepatopancreas. The mRNA levels of Wap65 were increased by *i. p.* injection of endotoxin, indicating that it may be also functional in self-defensive systems from infections as well as in acclimatory systems as shown in Fig. G-1.

Although remarkable insights into the mechanisms of temperature acclimation of goldfish were obtained from this investigation, exact functions of this protein remained

unclear. The possible functions of Wap65 are speculative in the present stage. Hazel and Prosser (1974) summarized that several enzymes in fish were enhanced their activity under the condition of warm temperature acclimation. The majority of these enzymes are associated with metabolism in lysosomes as well as those for urea and nitrogen and functional in the breakdown of metabolic products. It was hypothesized that these changes in enzymic activity may be adaptive with respect to specific functions such as the elimination of potentially toxic products in metabolism. In this context, it seems plausible to postulate that Wap65 is functional in scavenging of toxic products. Although Wap65 showed no affinity for heme-agarose in Chapter 4, these results do not eliminate the possibility of the heme binding property of Wap65. The method adopted was specialized for isolation of mammalian hemopexins, and other heme-binding proteins such as albumin could not be isolated under the conditions described in Chapter 4 (Muller-Eberhard, 1983). If Wap65 has a heme transport-property, the protein should be called as a goldfish hemopexin, then following hypothesis is possible; one of the primary roles for hemopexin is to prevent cells from heme-catalyzed oxidative damages (Gutteridge *et al.* 1988, Vincent *et al.* 1988). The presence of free heme may be more harmful in warm than cold body for poikilotherms. The increased abundance of heme-binding proteins such as hemopexin in accordance with increased body temperature may enhance the efficient removal of heme from circulation, giving an adaptive potential for increasing environmental temperatures. Albumin, the most

abundant protein in mammalian plasma, known to have functions as a depositor and transporter and responsible for decreasing toxicity of bilirubin and many cations (Kragh-Hansen, 1981). Concerning the amount of Wap65 in plasma, it would be consistent with the speculation of Hazel and Prosser (1974) to consider that a function of Wap65 might be similar to that of mammalian albumin. The other speculation is that compensatory adjustment in the oxygen-carrying capacity of the blood after warm temperature acclimation involves the activation of Wap65 transcription. Houston and Cry (1974) reported a significant increase in the total hemoglobin concentration in goldfish in association with acclimation to an increased temperature. Such response was considered to facilitate fish to adapt to an relatively hypoxic environment due to the effect of elevated temperature on oxygen solubility. It is possible that the hemoglobin-associated regulations include these of heme-binding protein responsible for heme turnover.

Comparison of Wap65 with mammalian hemopexins revealed significant similarities in their structures and regulations of gene expression. However, there must be many unknown functions of Wap65 in the warm acclimation of fish, which have probably been established in accordance with the acquisition of eurythermal nature in the process of evolution. Alternatively, such function of fish could have been lost in mammals due to their homoiothermal potential. To evaluate the function of Wap65 and regulation of its expression will be helpful for understanding the molecular mechanisms of temperature acclimation of fish.

Summary

Chapter 1. Changes in protein composition in goldfish following temperature acclimation.

Goldfish *Carassius auratus* were acclimated to either 10 or 30°C, and their protein compositions in several tissues were analyzed electrophoretically. Two-dimensional electrophoresis employing isoelectric focusing and SDS-PAGE revealed the increased abundance of a 65-kDa protein in muscle in 30°C-acclimated fish, while a 55-kDa protein was predominant in the 10°C-acclimated counterparts. Goldfish brain and hepatopancreas tissues together with plasma exhibited similar changes with respect to 65-kDa protein. These results suggest that the 65-kDa proteins are distributed to various tissues of warm temperature-acclimated fish.

Chapter 2. Specification of the warm temperature acclimation-related 65-kDa protein, Wap65, in goldfish.

The 65-kDa protein specific to the 30°C-acclimated fish was extracted with 70% formic acid from the gel after two-dimensional electrophoresis of the muscle extract. The 65-kDa protein thus prepared to homogeneity was used to raise specific antibodies in rabbit by conventional methods. The antibody produced exhibited specific cross-reaction with a protein having the same molecular weight from brain, hepatopancreas and plasma, suggesting that the 65-kDa protein is a ubiquitous component in the 30°C-acclimated goldfish. When water temperature was increased from 20 to 30°C over a 20

h period, a prominent amount of the 65-kDa protein was observed in muscle tissue extracts within 5 days of additional rearing. These results were obtained by immunoblot analysis with the specific antibody. The N-terminal amino acid sequence of the 65-kDa protein was determined as Asp-Glu-Pro-Gln-Gly-His-Gln-His(or Asp)-Glu-Leu, differing from those of a family of known heat shock proteins having about 70 kDa in a molecular mass. Thus, the 65-kDa protein was named Wap65 for its meaning of the warm temperature acclimation-related 65-kDa protein.

Chapter 3. Isolation of goldfish Wap65.

Wap65 was purified from epaxial muscle of 30°C-acclimated goldfish by a series of chromatographic steps using ion-exchange, gel filtration, and reversed-phase columns while monitoring immuno-reaction with specific antibody. The purified Wap65 gave one band in SDS-PAGE. The molecular weight of the protein was determined to be 65,000 by gel filtration and SDS-PAGE, demonstrating that it consists of a single polypeptide chain. Forty-four amino acid residues were determined by N-terminal amino acid sequencing. The sequence was fairly rich in histidine and phenylalanine. Homology research revealed that Wap65 has 29 and 26% homology to the spinach RNA polymerase β' subunit and human tyrosine hydroxylase, respectively

Chapter 4. cDNA cloning of goldfish Wap65.

cDNAs encoding Wap65 were cloned from the muscle and

hepatopancreas cDNA libraries of the 30°C-acclimated goldfish, and their nucleotide sequences containing 5' and 3' non-coding regions together with their polyadenylation signal were determined. The deduced amino acid sequence of Wap65 was 31% homologous to rat hemopexin. However, goldfish Wap65 lacked a few possible glycosylation sites and presumed functional histidine residues, implying that it may have different functions from hemopexin. Wap65 contained a leader peptide of 30 amino acids and a mature protein region of 415 amino acids. Southern blot analysis demonstrated that the protein is expressed by a single copy gene in the goldfish haploid. In RNA blot analysis using isolated cDNA clones, a single transcript of about 2.0-kilo bases was detected in the hepatopancreas, but not in brain, muscle and hemocytes. The abundancy of this transcript markedly increased in the hepatopancreas as a result of warm temperature acclimation. Electrophoretic analysis of plasma proteins revealed a good correlation of plasma Wap65 levels to those of the corresponding transcript in the hepatopancreas, suggesting that serum Wap65 concentrations are regulated mainly by transcript levels in the hepatopancreas *via* the secretion process.

Chapter 5. Structure and regulation of goldfish Wap65 gene, *wap65*.

The expression of the Wap65 gene, *wap65*, was examined for hepatopancreas, intestine, ovary, heart, gill, eye, skin, hemocytes, muscle and brain from the 30°C-acclimated goldfish

by reverse transcription PCR, revealing hepatopancreas-specific expression of the gene. In Northern blot analysis, levels of Wap65 mRNA increased after raising water temperature fr 10 to 30°C. This increase reached a maximum of 40-fold on day 3 after following temperature shift. On day 21 after temperature shift, levels were 10-fold compared with those of the 10°C-acclimated fish. In the case of lowering water temperature from 30 to 10°C, levels of Wap65 mRNA were gradually decreased with levels reaching 1/10 on day 21 in comparison to those o initial fish. The 5' region of *wap65* was cloned from genomic library of goldfish and its nucleotide sequence containing a putative promoter region and 4 introns was determined. Transcription start points were determined by primer extension analysis referring to the sequencing products of the cloned *wap65*. The 5' flanking region and intron of *wap65* contained enhancer motifs including cytokine responsive lements known in mammals. This suggests that some of these elements are involved in the transcriptional regulation of *wap65*. LPS administration into the 10°C-acclimated goldfish resulted in the accumulation of Wap65 mRNA in hepatopancreas, imolying the involvement of immune systems-associated regulation in the transcription of Wap65.

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Figures

Fig. 1-1. SDS-PAGE patterns of the muscle extract from goldfish acclimated to either 30 or 10°C. In the photograph are shown patterns of three individuals each for 30°C- and 10°C-acclimated fish. The upward arrow indicates the 65-kDa protein dominating in the 30°C-acclimated goldfish.

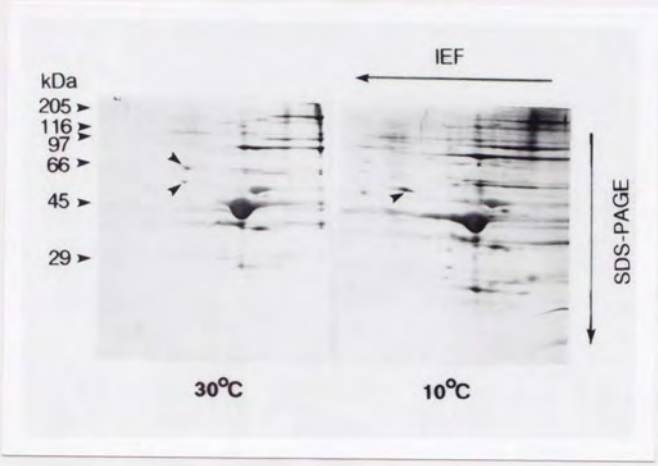


Fig. 1-2. Two-dimensional electrophoretic patterns of the muscle extract from goldfish acclimated to either 30 or 10°C. The downward arrowhead indicates the 65-kDa protein dominating in the 30°C-acclimated fish, whereas upward arrowheads indicate the 55-kDa protein dominating in the 10°C-acclimated fish. Numbers in the left extreme of the panel indicate molecular masses of standard proteins.

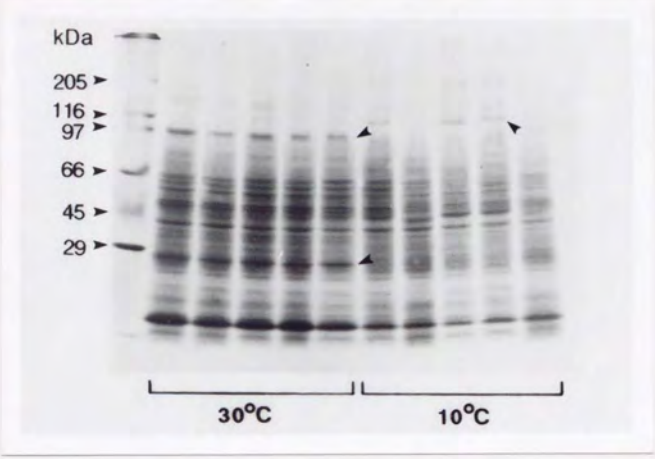


Fig. 1-3. SDS-PAGE patterns of the hepatopancreas extract from goldfish acclimated to either 30 or 10°C. The panel shows patterns of 5 individuals each for 30°C- and 10°C-acclimated fish. Downward arrowheads in panel A indicate 97-kDa and 28-kDa proteins dominating in the 30°C-acclimated fish, while an upward arrowhead indicates a 120-kDa protein specific to female fish.

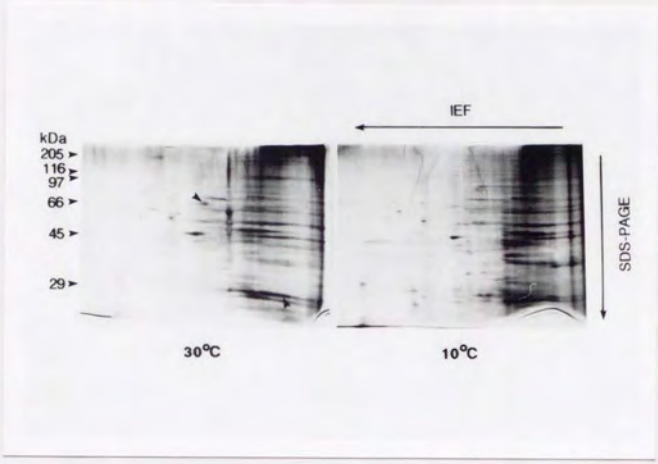


Fig. 1-4. Two-dimensional electrophoretic patterns of the hepatopancreas extrct from goldfish acclimated to either 30 or 10°C. Downward and a upward arrowheads indicate the 65-kDa and 28-kDa proteins, respectively, dominating in the 30°C-acclimated fish.

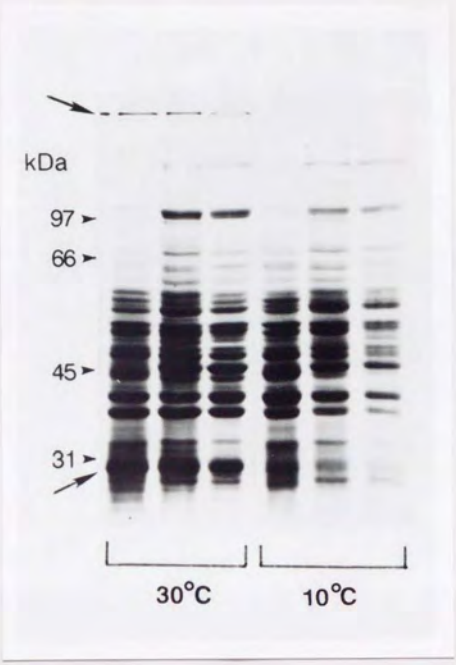


Fig. 1-5. SDS-PAGE patterns of the brain extract from goldfish acclimated to either 30 or 10°C. Upward and downward arrows indicate proteins having a molecular mass of 28 kDa and that of more than 200 kDa in the 30°C-acclimated fish, respectively. Numbers in the extreme left of the panel indicate molecular masses of standard proteins.

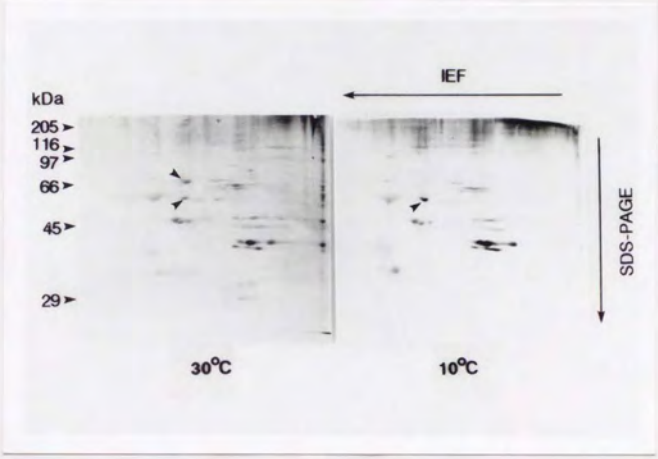


Fig. 1-6. Two-dimensional electrophoretic patterns of the brain extract from goldfish acclimated to either 30 and 10°C. Downward and upward arrowheads indicate the 65-kDa and 55-kDa proteins, respectively.

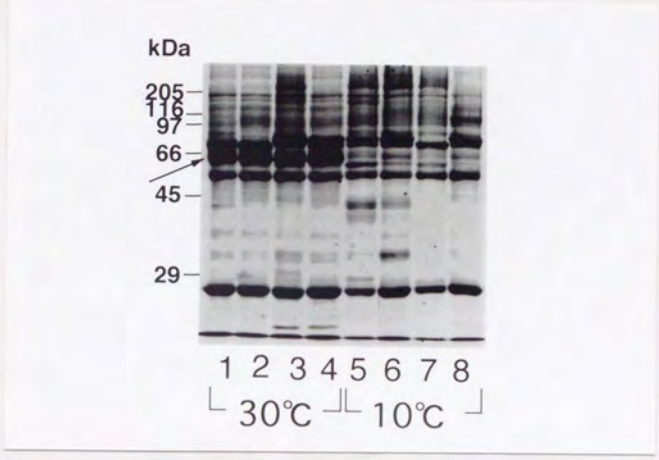


Fig. 1-7. SDS-PAGE patterns of plasma proteins from goldfish acclimated to either 30 or 10°C. In the panel are shown patterns of four individuals each for 30°C- and 10°C-acclimated fish. An arrow indicates the 65-kDa protein dominating in the warm-acclimated goldfish.

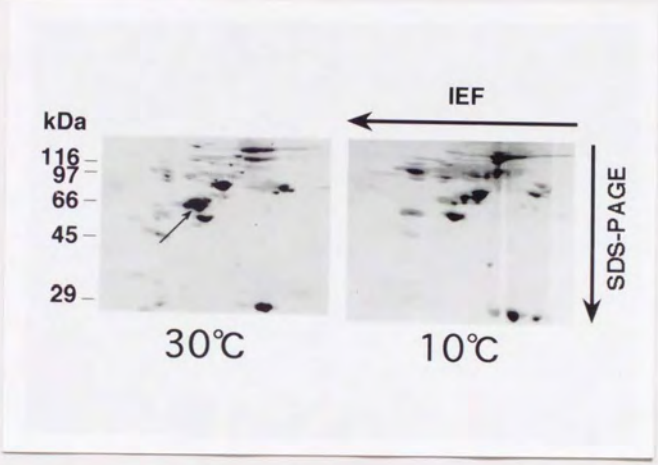


Fig. 1-8. Two-dimensional electrophoretic patterns of plasma proteins from goldfish acclimated to either 30 and 10°C. Upward arrowheads indicate the 65-kDa proteins, respectively.

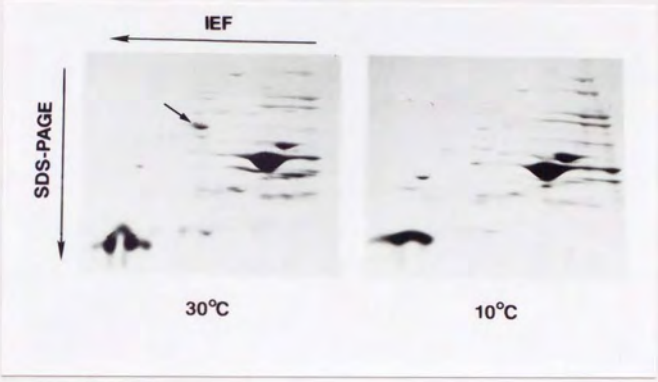


Fig. 2-1. Two-dimensional electrophoretic patterns of the muscle extract from goldfish acclimated to either 30 or 10°C. The arrow indicates the 65-kDa protein dominating in the 30°C-acclimated fish.

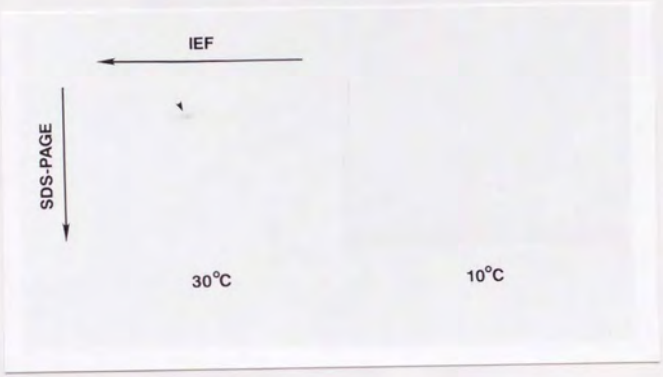


Fig. 2-2. Immunoblot patterns after two-dimensional electrophoresis of the muscle extract from goldfish acclimated to either 30 or 10°C. An arrowhead indicates the reactive protein having a molecular mass of 65 kDa and dominating in the 30°C-acclimated fish.

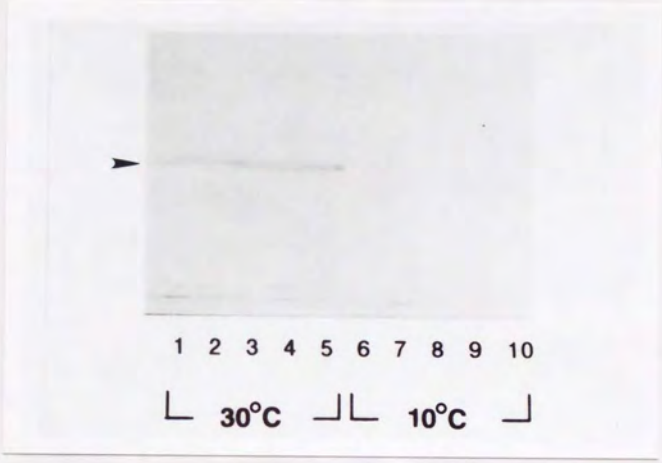


Fig. 2-3. Immunoblot patterns after SDS-PAGE of the brain extract from goldfish acclimated to either 30 or 10°C. An arrowhead indicates reactive protein bands having a molecular mass of 65 kDa and dominating in the 30°C-acclimated fish (lanes 1 - 5).

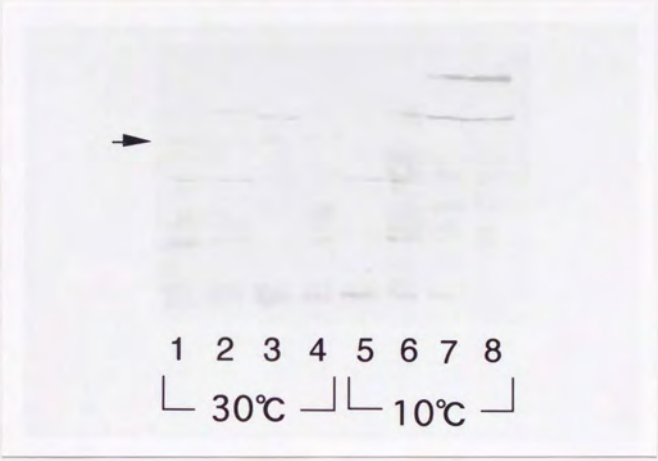


Fig. 2-4. Immunoblot patterns after SDS-PAGE of the hepatopancreas extract from goldfish acclimated to either 30 or 10°C. An arrow indicates reactive protein bands having a molecular mass of 65 kDa specific to the 30°C-acclimated fish (lanes 1 - 4).

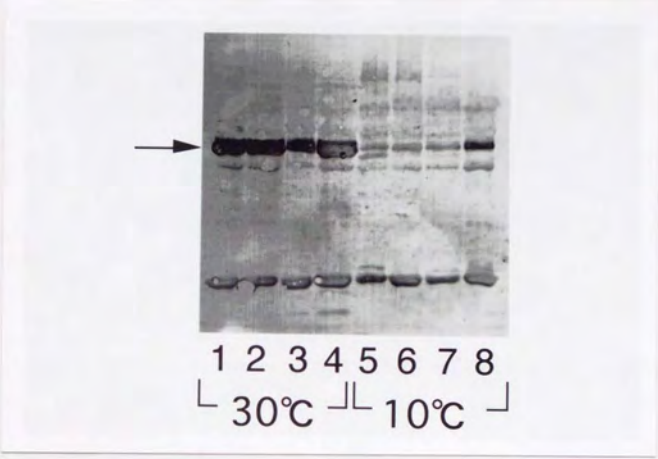


Fig. 2-5. Immunoblot patterns after SDS-PAGE of the plasm from goldfish acclimated to either 30 or 10°C. An arrow indicates reactive protein bands having a molecular mass of 65 kDa and dominating in the 30°C-acclimated fish (lanes 1 - 4).

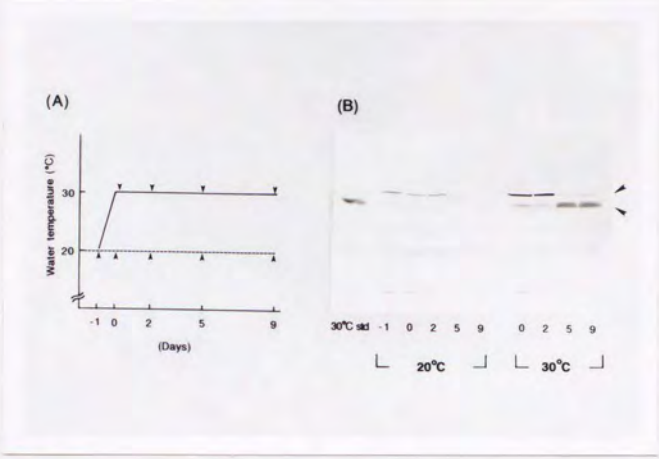


Fig. 2-6. The time schedule for raising water temperature of the goldfish aquarium (A) and its effect on immunoblot patterns after SDS-PAGE of the muscle extract from goldfish (B). Sample fish were taken at stages indicated by arrowheads in panel A, from the 20°C-aquarium or on day 0, 2, 5, and 9 after raising water temperature to 30°C. Control samples were taken at those stages from the 20°C-acclimated fish. A downward arrowhead in panel B indicates the reactive 70-kDa protein band. An upward arrowhead indicates the 65-kDa protein band which was increased in concentration on day 5 after raising water temperature. In panel B, the muscle tissue extract from the 30°C-acclimated fish was used as a control (30°C std).

```
G65  D E P Q G H Q H E L
                (D)

T    M S - K G P A V G I  D L G T T Y S C V
H    · A - · A A · · · ·  · · · · · · · · ·
Xl   · · T · · V · · · ·  · · · · · T · · ·
Ec   · G - · · - I I · ·  · · · · · N · · ·
```

Fig. 2-7. The N-terminal amino acid sequence of the 65-kDa (G65) protein from the muscle extract of the 30°C-acclimated goldfish in comparison with those of a family of 70-kDa heat shock protein. The sequence of Hsc70 from rainbow trout (T) (Zaharullah et al. 1992), and Hsp70 from human (H), *Xenopus laevis* (Xl) and *E. coli* (Ec) reviewed by Lindquist (1986) are shown. Only different amino acids are indicated and hyphens mark deletions.

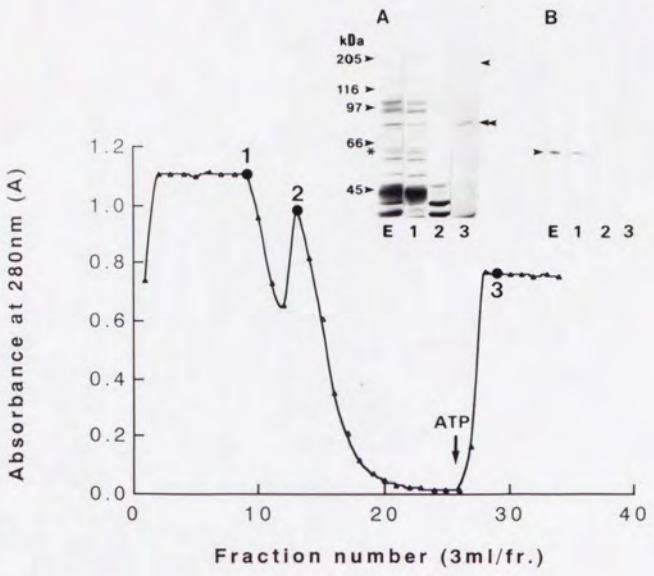


Fig. 2-8. The elution profile in ATP-agarose affinity chromatography of the muscle extract from goldfish acclimated to 30°C. Panels A and B represent SDS-PAGE and immunoblot patterns, respectively, for the muscle extracts (lane E) and typical fractions numbered in the elution curve. Single and double arrowheads in panel A indicate 200-kDa and 70-kDa proteins retained on the column in the absence of ATP, while a single arrowhead in panel B indicate the 65-kDa protein. fr., fraction

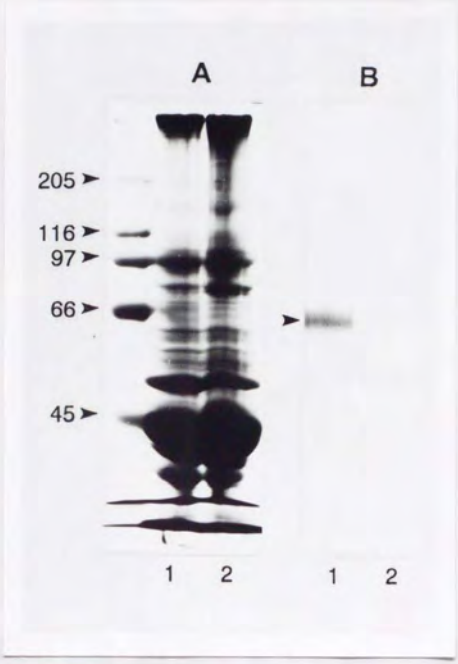


Fig. 3-1. SDS-PAGE (A) and immunoblot (B) patterns of the muscle extract from goldfish acclimated to 30 or 10°C. Proteins were extracted from epaxial muscle of goldfish acclimated to either 30 (lane 1) or 10°C (lane 2). An arrowhead in panel B indicates a protein band reactive with antibody specific to Wap65 appearing in 30°C-acclimated fish. Sample volumes used were 10 µl for SDS-PAGE.

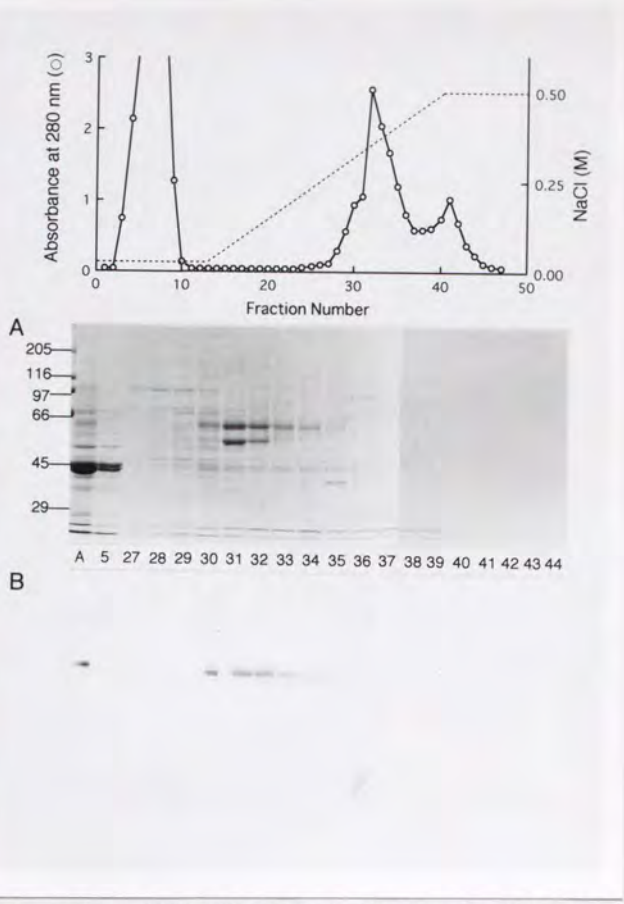


Fig. 3-2. TSKgel DEAE-5PW HPLC of the muscle extracts from goldfish acclimated to 30°C. The precipitate between 50 - 80% $(NH_4)_2SO_4$ saturation was dissolved in and dialyzed against buffer A containing 20 mM Tris-acetate (pH 7.5), 20 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and 0.05% NaN_3. The dialysate was then centrifuged at 33,000 x g for 20 min at 4°C. The supernatant containing Wap65 was applied to ion-exchange chromatography using a TSKgel DEAE-5PW column equilibrated with buffer A and proteins were eluted with a linear gradient of 20 - 500 mM NaCl. The flow rate was 1.0 ml/min. Fractions of 1.0 ml were collected and analyzed for their absorbance at 280 nm (○). In panel A are shown Coomassie Brilliant Blue-stained 12.5% SDS-PAGE patterns of selected fractions and in panel B, their immuno-staining patterns. The sample before chromatography was loaded in lane A. Sample volumes used were 10 µl except for lane A and fraction 5 where 1 µl of samples were loaded. In this and following figures, molecular weight markers (kDa) are indicated at the left.

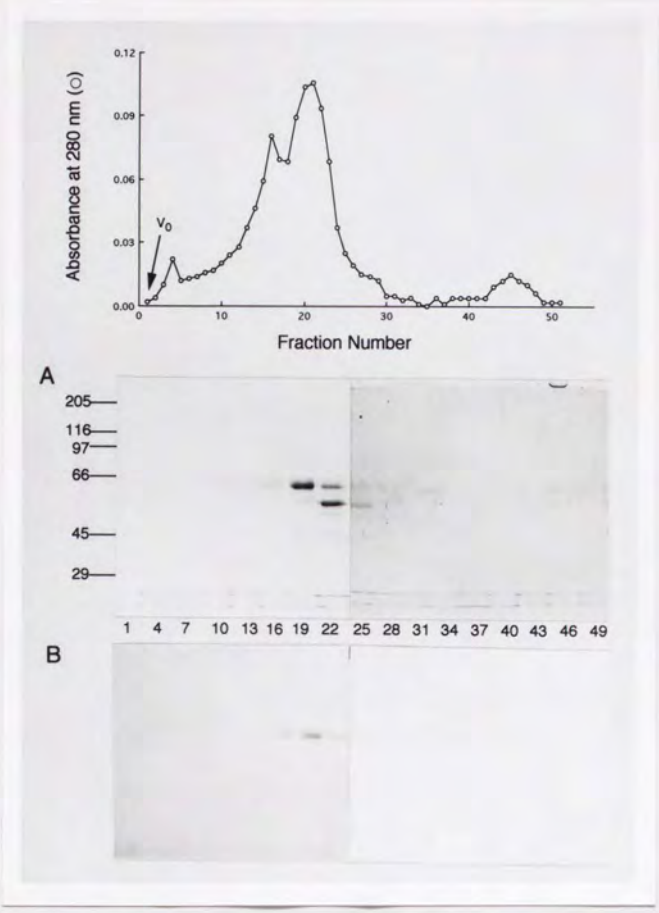


Fig. 3-3. Gel filtration of Wap65 fractions. Wap65 fractions eluted from a TSKgel DEAE-5PW column (fractions 31 - 34 in Fig. 3-2) were pooled and applied to a TSKgel G3000 SWG column equilibrated with buffer B containing 50 mM potassium phosphate (pH 6.9), 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, and 0.05% NaN_3. The flow rate was 3.0 ml/min. Fractions of 3.0 ml were collected and analyzed for their absorbance at 280 nm (○). In panel A are shown Coomassie Brrilliant Blue-stained 12.5% SDS-PAGE patterns for every three fractions and in panel B, their immuno-staining patterns. Sample volumes used were 10 µl for SDS-PAGE. V_0 means the void volume of the used column.

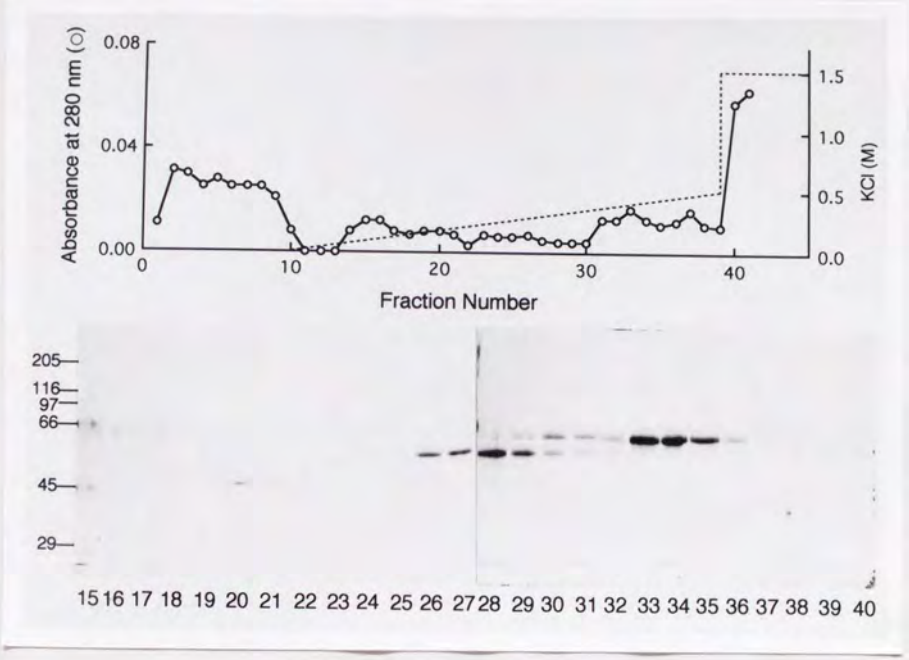


Fig. 3-4. DEAE-Toyopearl column chromatography of Wap65 fractions. Wap65 fractions eluted from a TSKgel G3000 SWG column (fraction 18 - 21 in Fig. 3-3) were pooled, dialyzed against buffer C containing 25 mM Tris-HCl (pH 8.0), 7 M urea, 15 mM β-mercaptoethanol, and applied to a DEAE-Toyopearl column equilibrated in the same buffer. A linear gradient with 300 ml of buffer C and 300 ml of buffer C plus 0.5 M KCl was used. The flow rate was 60 ml/h. Fractions of 5.0 ml were collected and analyzed for their absorbance at 280 nm (○). In the photograph are shown silver nitrate-stained 12.5% SDS-PAGE patterns of selected fractions. Sample volumes used were 10 µl for SDS-PAGE.

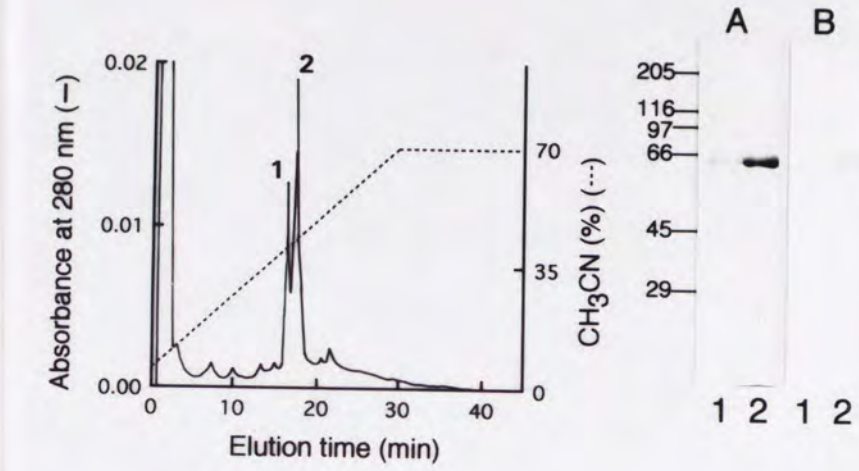


Fig. 3-5. TSKgel Phenyl-5PW reversed-phase HPLC of Wap65. Wap65 fractions eluted from a DEAE-Toyopearl (fraction 34 - 36 in Fig. 3-4) were dialyzed against 0.05% DTT for 24 h and lyophilized. The lyophilized materials were dissolved in 0.1 % TFA containing 8 M urea, then applied to a TSKgel Phenyl-5PW reversed-phase column equilibrated with 0.1% TFA. Proteins were eluted with a linear gradient of 0.7 - 70% acetonitrile containing 0.1% TFA. The flow rate was 1.0 ml/min. In panel A are shown Coomassie Brilliant Blue-stained 12.5% SDS-PAGE patterns and in panel B, their immuno-staining patterns for peak 1 (lane 1) and peak 2 (lane 2). One-third amounts of these fractions obtained after changing the eluting buffer to buffer A containing 20 mM Tris-acetate (pH 7.5), 20 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF and 0.05% NaN_3 were loaded onto SDS-PAGE.

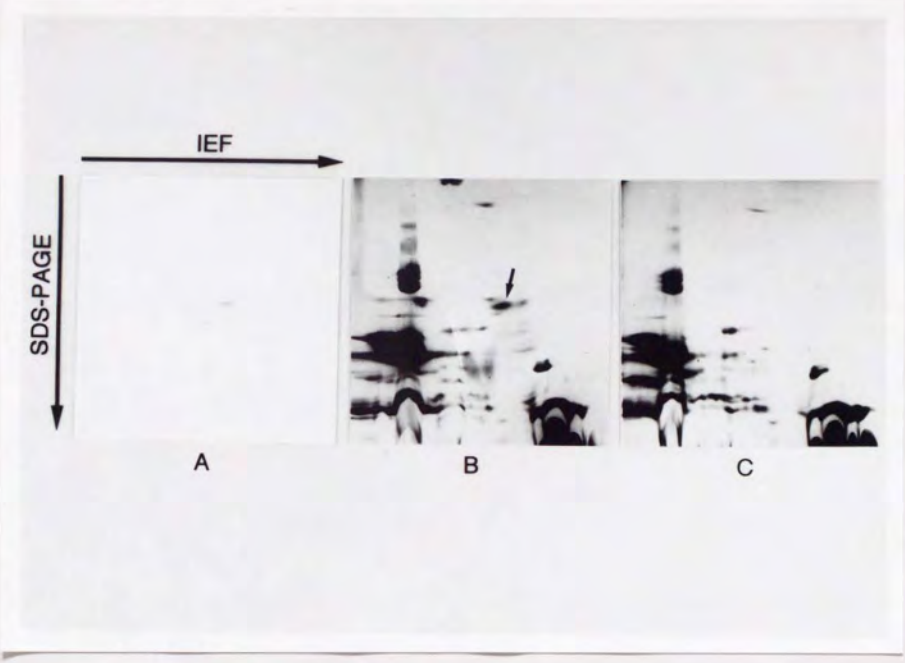


Fig. 3-6. Two-dimensional electrophoretic patterns of Wap65 (A) and the muscle extract from goldfish acclimated to either 30 (B) or 10°C (C). Wap65 was obtained as peak 2 in TSKgel Phenyl-5PW HPLC of Fig. 3-5. An arrow in panel B indicates Wap65 dominating in the 30°C-acclimated fish. Portions of 100 µl extract were used in panels B and C.

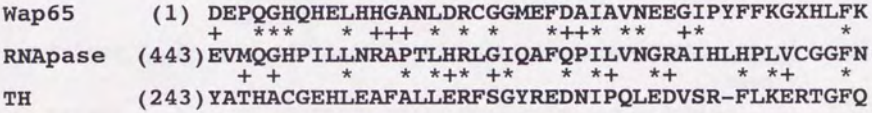
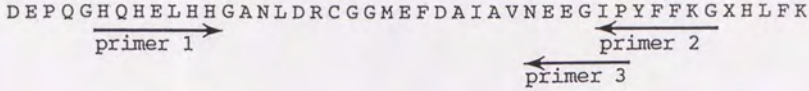


Fig. 3-7. The N-Terminal amino acid sequence of Wap65 in comparison with sequences of spinach RNA polymerase β' subunit and tyrosine hydroxylase. The numbers start from the N-terminus. An x at position 41 from the N-terminus of Wap65 indicates an unidentified amino acid. Identical and conserved amino acids between Wap65 and RNA polymerase β' subunit (RNApase β') or tyrosine hydroxylase (TH) are indicated by asterisks and plusses, respectively. A dash denotes a gap introduced to maximize homology.



Primer 1 5' - GAATTCCA(TC)CA(AG)CA(TC)GA(AG)CT(TCAG)CA(TC)CA - 3'
(*Eco*RI)

Primer 2 3' - TA(AGT)GG(AGTC)AT(AG)AA(AG)AA(AG)TT(CT)CCTAGG - 5'
(*Bam*HI)

Primer 3 3' - TT(AG)CT(CT)CT(CT)CC(AGCT)TAAGT GGCCTAAG - 5'
(*Bam*HI)

Fig. 4-1. The N-terminal amino acid sequence of Wap65 and nucleotide sequences of synthesized DNA primers for PCR. The N-terminal amino acid sequence was determined for Wap65 purified from the 30°C-acclimated goldfish muscle in Chapter 3. An X at position 41 from the N-terminus indicates an unidentified amino acid. The primers contain all possible combinations of nucleotides that encode respective amino acids except for primer 1, where one of the triplets for leucine, TT(AG), was omitted.

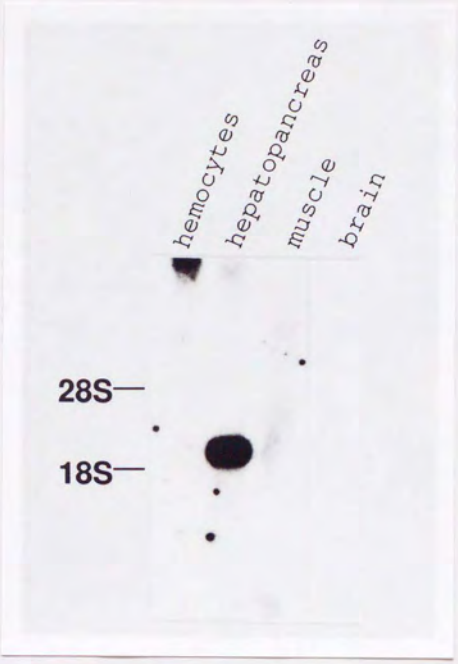


Fig. 4-2. Northern blot analysis of Wap65 mRNA in hemocytes, hepatopancreas, muscle and brain from the 30°C-acclimated goldfish. Ten µg of total RNAs from each tissue were subjected to electrophoresis. The blots were probed with pw65-N DNA.

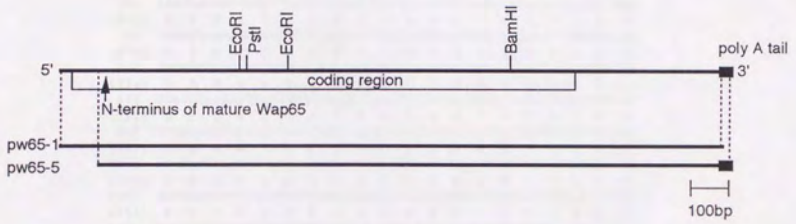


Fig. 4-3. A partial restriction endonuclease map and two cDNA clones coding for Wap65. An arrow indicates a site of the codon coding for aspartic acid which occurs at the N-terminus of Wap65 isolated from the 30°C-acclimated goldfish.

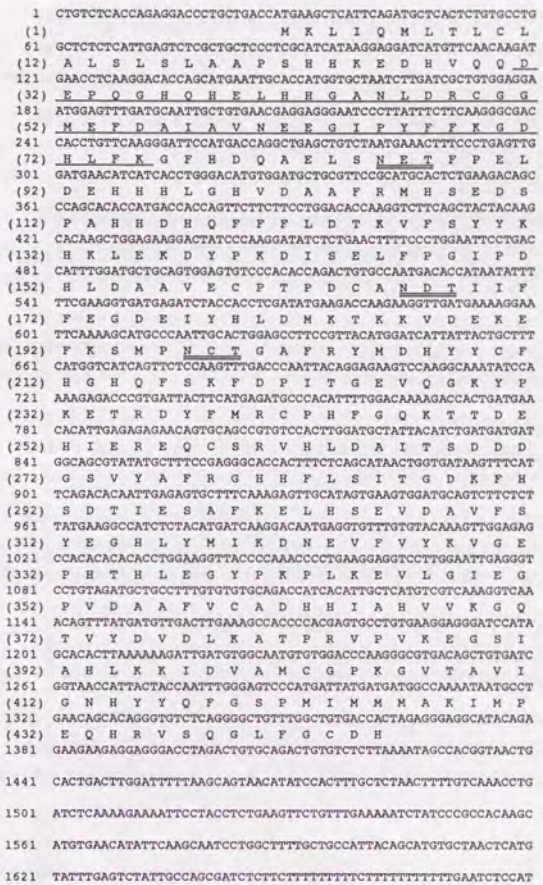


Fig. 4-4. DNA nucleotide and deduced amino acid sequences of Wap65. The partial amino acid sequence directly determined for Wap65 purified from the muscle is underlined. Potential N-linked glycosylation sites are double-underlined. A putative polyadenylation signal is boxed.

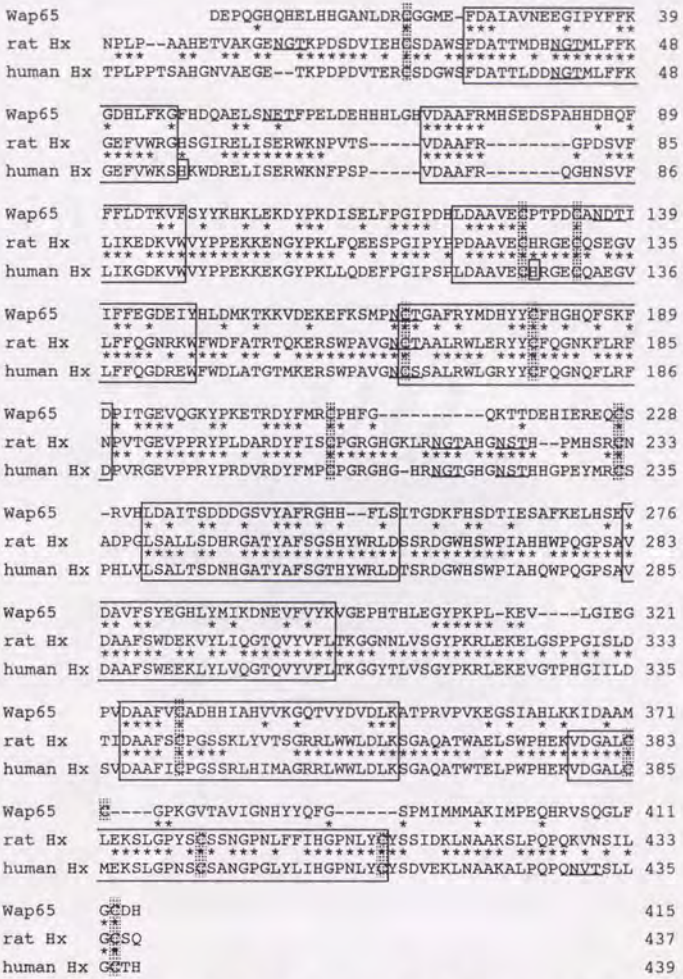


Fig. 4-5. Comparison of the amino acid sequence of Wap65 with those of rat and human hemopexins. The number starts from the N-terminal amino acid of matured proteins. Identical amino acids between Wap65 and rat hemopexin (Hx) or between rat and human hemopexin are indicated by asterisks. Dashes denote gaps introduced to maximize homology. The conserved cysteine residues are meshed, whereas the conserved histidine residues, which are assumed to serve as heme axial ligands in human hemopexin, are boxed. Potential N-linked glycosylation sites are underlined. Internal repeats characteristic of pexin gene family (Jenne and Stanley, 1987) are boxed. Amino acid sequences of human and rat hemopexin are cited from Takahashi et al. (1985) and Nikkila et al. (1991), respectively.

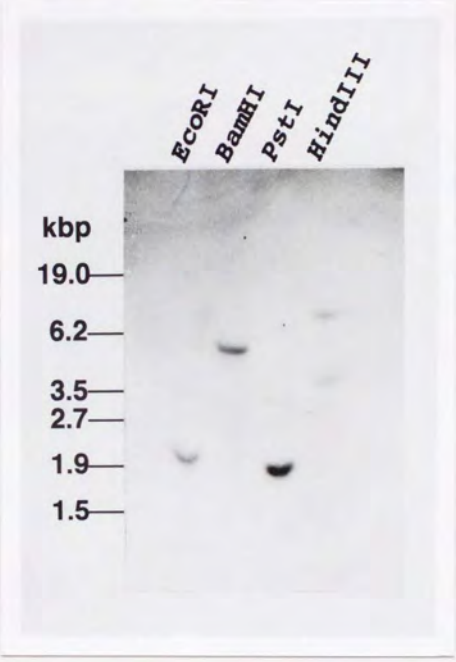


Fig. 4-6. Southern blot analysis of the Wap65 gene. Goldfish hepatopancreas genomic DNAs (10 µg/lane) were digested with a series of restriction endonucleases, electrophoresed in a 0.7% agarose gel, and transferred to a nylon membrane. A probe consisting of 217 bp was obtained after digestion of the Wap65 cDNA clone, pw65-1, with *Eco*RI. Molecular weight markers are *Eco*T14I digests of lambda phage DNA.

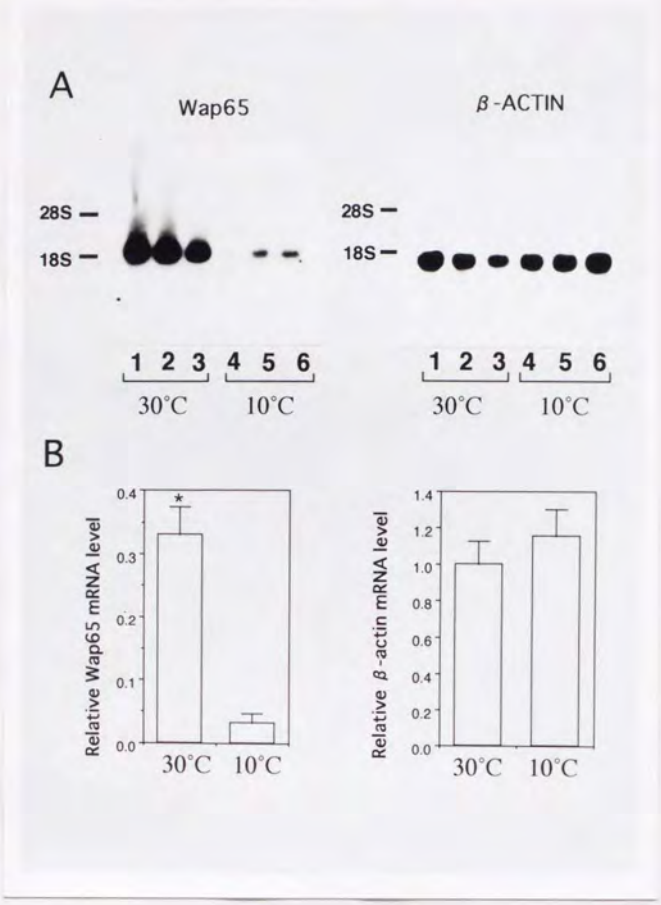


Fig. 4-7. Wap65 mRNA levels in hepatopancreas from goldfish acclimated to either 30 or 10°C. A, Northern blot analysis for three individuals each from goldfish acclimated to 30°C and 10°C. Lanes 1 - 3 contain 10 µg of total RNAs from fish acclimated to 30°C, whereas lanes 4 - 6 contain those from fish acclimated to 10°C. RNAs were electrophoresed in 0.7% agarose gels, transferred to nylon membranes, and hybridized with randomly labeled pw65-1 [^{32}P] DNA or with that coding for goldfish β-actin. B, relative transcriptional levels of Wap65 and β-actin in hepatopancreas. RNA blots were quantified with a Fujix BAS1000 densitometer.

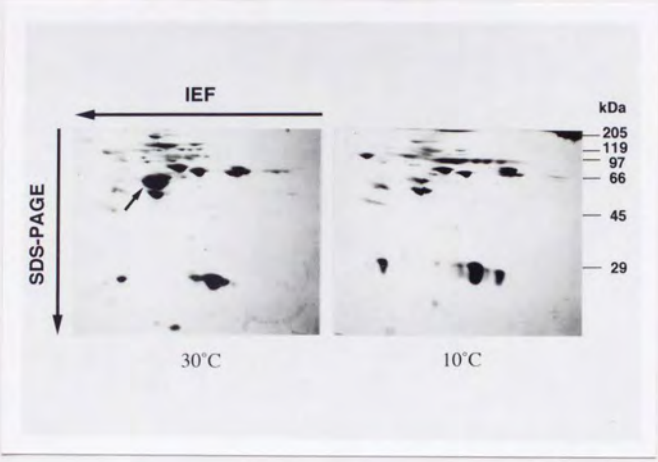


Fig. 4-8. Two-dimensional electrophoretic patterns of plasma proteins from goldfish acclimated to either 30 or 10°C. An arrow indicates Wap65 dominating in the 30°C-acclimated fish. Protein spots on gels separated by two-dimensional electrophoresis were subjected to N-terminal amino sequencing or quantified with a Shimadzu CS-9300PC densitometer.

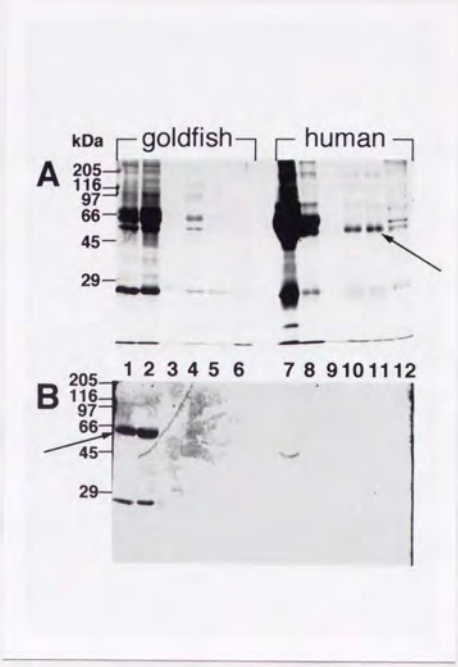


Fig. 4-9. SDS-PAGE (A) and immunoblot (B) patterns for the typical fractions in heme affinity chromatography for plasma protein from the 30°C-acclimated goldfish and human. Plasma of the 30°C-acclimated goldfish (lane 1 - 6) and human (lane 7 - 12) were applied on the column. An arrow in panel B indicates Wap65, while an arrow in panel A indicates a human 60-kDa protein showing affinity for the column. Lanes 1 and 7, plasmas of goldfish and human, respectively; lanes 2, 3, 8 and 9, fractions eluted with 10 mM sodium phosphate buffer (pH 7.5) containing 0.5 M NaCl; lanes 4, 5, 10 and 11, fractions eluted with 0.2 M sodium acetate buffer (pH 5.2); lanes 6 and 12, 5 μg of heme agarose resin after washing with 0.2 M sodium acetate buffer (pH 5.2).

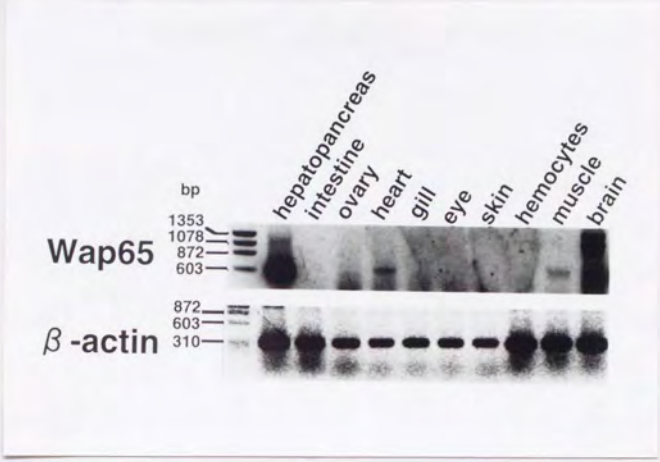


Fig. 5-1. RT-PCR amplification of Wap65 mRNA from different tissues. Total RNA was isolated from hepatopancreas, intestine, ovary, heart, gill, eye, skin, hemocytes, muscle and brain of the 30°C-acclimated goldfish. After PCR, amplified products were analyzed in agarose gels stained with ethidium bromide before the negative image was recorded. The numbers, in base pairs, of a ϕx174 digested with *Hae*III are shown in the left sides of the gels.

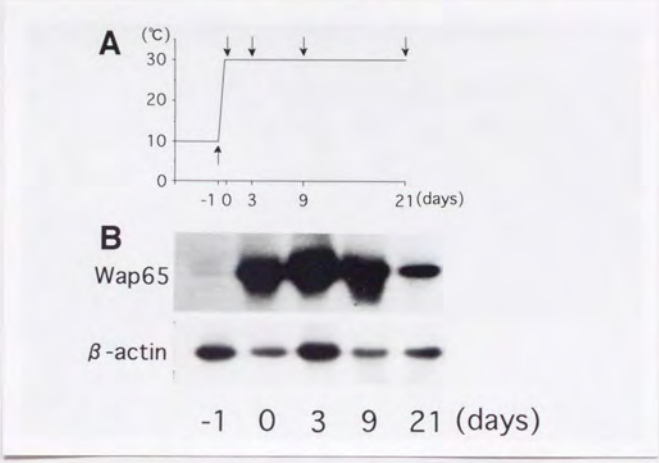


Fig. 5-2. The time schedule for raising water temperature of the aquarium (A) and its effect on Northern blot patterns of Wap65 mRNA and β-actin mRNA in hepatopancreas of goldfish(B). Sample fish were taken at stages indicated by arrows in panel A, from the 10°C-aquarium or on day 0, 3, 9 and 21 after raising water temperature to 30°C. Panel B shows representative results obtained with four fish in each acclimation condition.

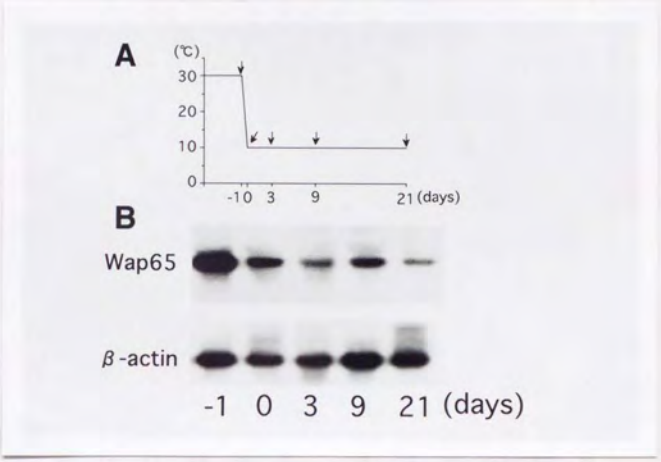


Fig. 5-3. The time schedule for lowering water temperature of the aquarium (A) and its effect on Northern blot patterns of Wap65 mRNA and β-actin mRNA in hepatopancreas of goldfish(B). Sample fish were taken at stages indicated by arrows in panel A, from the 30°C-aquarium or on day 0, 3, 9 and 21 after lowering water temperature to 10°C.

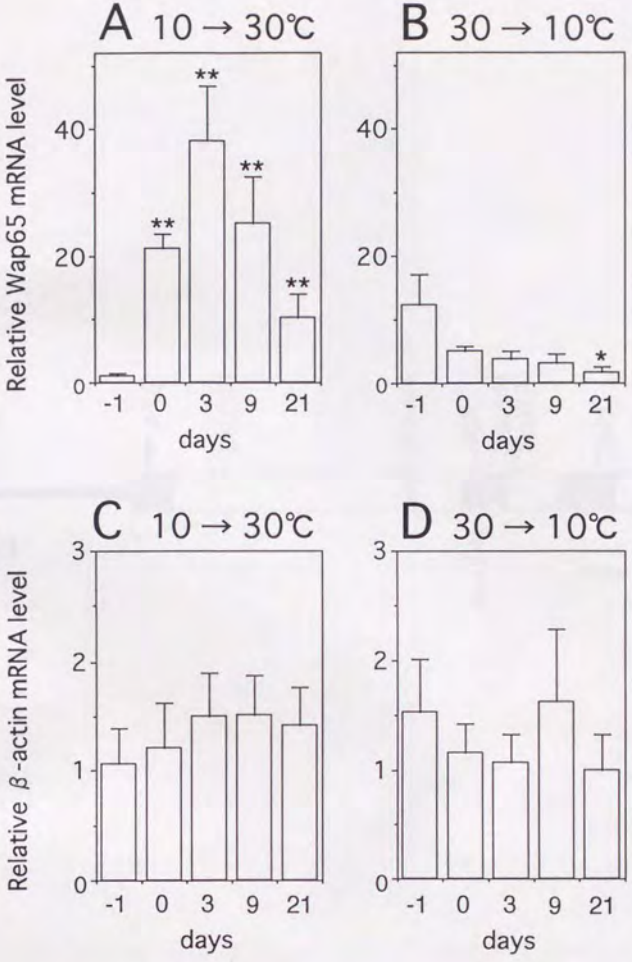


Fig. 5-4. Changes in relative mRNA levels of Wap65 (A, B) and β-actin (C, D) in hepatopancreas of goldfish following water temperature shifts. RNA blots were quantified with a Fujix BAS1000 densitometer as described in the text. The data shown in panels A and B are expressed in relation to the abundance of Wap65 mRNA in a 10°C-acclimated fish taken on -1 day in panel A. The data of β-actin were also standardized to the abundance of β-actin in the same fish. * ($p < 0.05$) and ** ($p < 0.01$) indicate the levels of significant differences compared with those of initial groups.

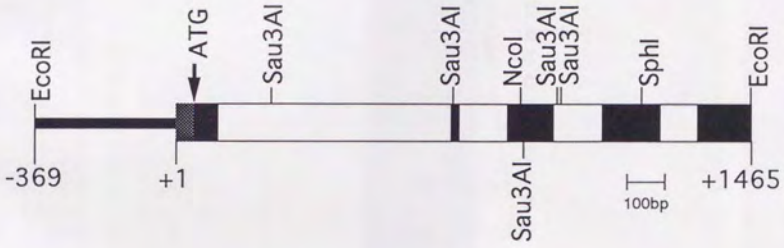


Fig. 5-5. Structure of the 5' region of the Wap65 gene. Various DNA regions of pGE-1 are depicted on the restriction map of the genomic DNA; a narrow bar for the region upstream from transcription start point; blank boxes for introns; shaded and solid boxes for untranslated and translated parts of exons, respectively. The transcription start point and the putative translation start codon were marked as +1 and ATG, respectively.

Fig. 5-6. Primer extension analysis of Wap65 mRNA. The extension products were separated on a 6% polyacrylamide gel containing 6 M urea. Lane 1, an extension product of hepatopancreatic RNA from the 30°C-acclimated goldfish; lane 2, an extension product from fish taken 3 days after raising water temperature from 10 to 30°; lanes G - T, the four sequencing reactions for the pGE-1 using extension oligonucleotide primer terminated with dideoxynucleotides.

Fig. 5-7. The nucleotide sequence of the 1.8-kbp *Eco*RI fragment (pGE-1) containing the 5' end of goldfish *wap65*. The nucleotide assigned +1 is the transcription start point for the 30°C-acclimated fish. The reversed CAAT box and TATA box are boxed. Putative binding sites with transcription factors referring to vertebrate genes are underlined.

NF-IL6 site

```
consensus                    TKNNGNAAK
human      -124 TATTTGCAG   TGATGTAAT   CAGCG -102
                 | |  ||    ||  |  ||     |
goldfish   -133 GTTGTTTAG   TGTGGGCAT   GTGAC -111
                |  |        |  || ||    | | |
           -99  TCCTACGCT   TTGTGCAAA   CGGGG -77
```

Hemopexin IL-6-RE

```
consensus             CCGGGAA
human      -175 CTG   CCGGGAA   AAGGAGTCTCTTGGA -152
                |     |||||     |       | ||  |
goldfish   -168 TTC   AGGGGAA   GCGTGCAAACGTGCA -145
```

Fig. 5-8. Sequence comparison of the proximal promoter region of *wap65* with NF-IL6 and IL-6-RE in human hemopexin gene. Two regions of *wap65* gene are aligned with corresponding sequences of NF-IL6 and IL-6-RE in human hemopexin gene with their position numbers from the transcription start site. Mammalian consensus sequences are also aligned. Identical nucleotides are indicated by slashes. Matching nucleotides to the consensus sequences are shaded. The sequences of human hemopexin gene were cited from Altruda *et al.* (1988). The nucleotide is as follows: N, either A, C, G or T; K, G or T.

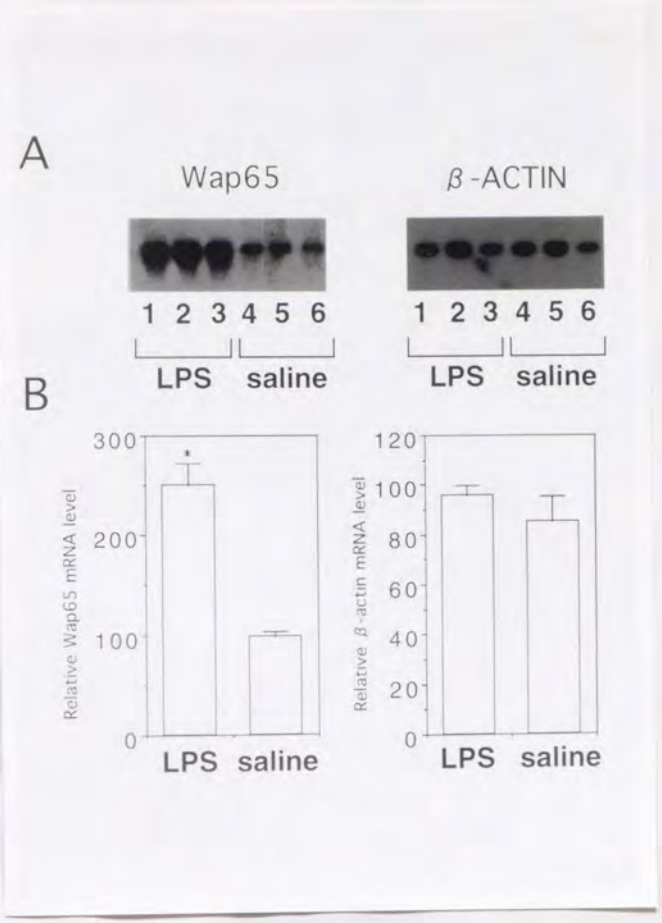


Fig. 5-9. Changes in Wap65 mRNA levels in hepatopancreas from the 10°C-acclimated goldfish following *i. p.* injection of LPS. A, Northern blot analysis for three individuals each from the 10°C-acclimated fish following *i. p.* injection of LPS and saline, respectively. Lanes 1 - 3 contain 10 μg of total RNAs from fish receiving an *i. p.* injection of LPS, whereas lanes 4 - 6 contain those of saline. RNAs were electrophoresed in 0.7% agarose gels, transferred to nylon membranes, and hybridized with randomly labeled pw65-1 [^{32}P] DNA. * ($p < 0.05$) indicates the levels of significant differences compared with those of saline injected groups. B, Relative mRNA levels of Wap65 and β-actin in hepatopancreas. RNA blots were quantified with a Fujix BAS1000 densitometer.

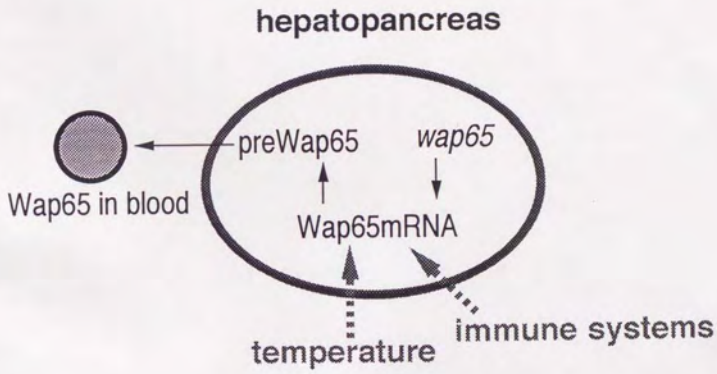
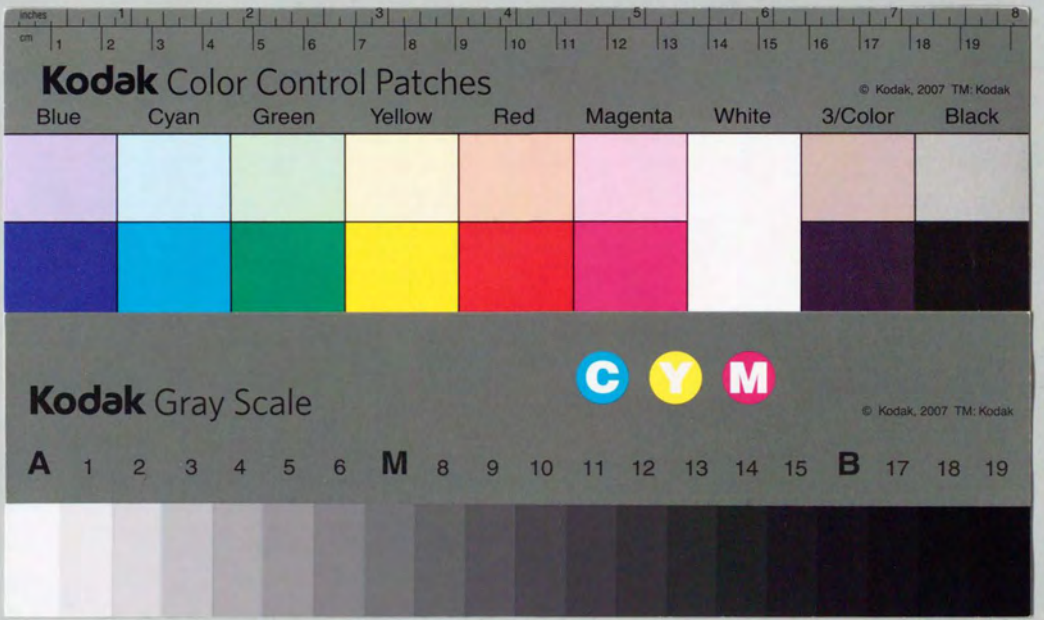


Fig. G-1. A regulation of Wap65 production in the goldfish. Warm water temperature and *i. p.* injection of LPS resulted in accumulation of Wap65 mRNA in hepatopancreas of goldfish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Red
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