Introduction

Thyroid hormones influence a wide range of biological activities. They affect numerous aspects of mammalian development and metabolism (Greenspan, 1994; for review see Utiger, 1995). They also play an important role in the life of non-mammalian vetebrates including the sloughing cycle of a snake (Chiu and Lam, 1994), changes during metamorphosis and the transition from aqueous to terrestrial life in amphibians (for reviews see Hourdry, 1993; Su *et al.*, 1999), as well as the development and differentiation during smoltification in fish (Yamano *et al.*, 1991; Miwa *et al.*, 1992; Miwa and Inui, 1991). The two major forms of thyroid hormones with significant biologic activity in vertebrates are L-3,5,3′,5′ - tetraiodothyronine (L-thyroxine, T4) and L-3,5,3′-triiodothyronine (L-triiodothyronine, T3). T3 is the thyroid hormone of higher biological activity (Samuels *et al.*, 1989; Surks and Oppenheimer, 1977), while T4 is the primary secretory product of the thyroid gland and functions as a prohormone (Puymirat, 1992; Surks *et al.*, 1973; Greenspan, 1994; Utiger, 1995).

Thyroid hormone action and metabolism occur in the intracellular compartment. The passage of T3 and T4 across cell membrane was suggested to depend on lipid composition of the membrane (Chehin *et al.*, 1999). However, as thyroid hormones have a strong tendency to partition into lipid membrane (Hillier, 1970; Dickson *et al.*, 1987), could result in marked depletion of thyroid hormones from blood (Mendel *et al.*, 1987). Thyroid hormone-binding proteins, thus, have been suggested to play an important role in determining the distribution of the hormones between aqueous and lipid phase of membranes (for review see Schreiber and Richardson, 1997).

Transthyretin (TTR) is one of the three thyroid hormone-binding proteins found in the plasma of larger mammals. It was first found in both serum and cerebrospinal fluid (CSF) (Kabat *et al.*, 1942 a, b), and was formerly referred to as thyroxine-binding prealbumin (Ingbar, 1958; Pages *et al.*, 1973). Its name was changed to TTR to indicate its two known properties, namely the binding of thyroid hormones and of retinol-binding protein (RBP) (Kanai *et al.*, 1968), the specific carrier of the alcohol form of vitamin A.

TTRs are widely distributed among species. It is a tetramer of identical subunits (Blake *et al.*, 1978) and has a molecular mass of 55 kDa. In human, TTR subunit consists of 127 amino acid residues (Kanda *et al.*, 1974). A large central channel with two sterically equivalent thyroid hormone binding sites, which differ in their relative binding affinity, is formed as the consequence of the tetrahedral arrangement of the TTR subunit (Blake *et al.*, 1978). Only one binding site of TTR is occupied by thyroid hormone under physiological

conditions (Pages *et al.*, 1973; Nilsson *et al.*, 1975). Although all amino acid residues reported to be participating in the binding of thyroid hormones in human TTR are conserved, the binding affinity to thyroid hormones varied among animal species during evolution (Chang *et al.*, 1999). During the evolution of mammalian TTRs from TTR ancestors, the affinity to thyroxine increased while the affinity to triiodothyronine decreased.

Recently, TTR were isolated and its cDNAs were cloned and sequenced for more than 25 vertebrate species, including mammalian, avian, reptilian, amphibian and fish (for reviews see Schreiber and Richardson, 1997; Schreiber *et al.*, 1999). Structural analysis of the TTR genes showed that the changes in TTR structure appearing during and persisting throughout evolution are concentrated at the N-terminal region of the protein. While the sequences of the amino acid residues that are involved in the binding interaction of TTR and thyroid hormones have not been altered since, at least, 400 million years ago. Pronounced changes in the primary structure of TTR occurred during evolution within the first 10 amino acids from the N-terminus. The N-terminus segment is longer and more hydrophobic in avian, reptilian, amphibian and fish than in mammalian TTRs. Three (for birds, reptiles, amphibian and fish) and two (for some marsupials) additional amino acids (compared with human TTR) are found in this region. In the three-dimensional structure of TTR, the N-terminal segment protrudes from the protein tetramer and is located near the entrance to the central channel, harboring the thyroid hormone binding site.

The correlation of changing of TTRs in structure and function, namely the binding affinities to thyroid hormones, during evolution of vertebrates, as well as the location in molecule of their N-termini have brought to the postulation that the changes in length and hydropathy of the N-termini of TTR subunits play role in changing the accessibility of the central channel for thyroid hormones. This study aims to elucidate the postulation by determining the changing of binding affinities to thyroid hormones of chimeric TTRs with variations in the N-terminal region, using crocodile TTR as a model. To gain insight, recombinant crocodile TTR and its chimeras were produced in *Pichia pastoris* using the methanol-inducible expression vector, pPIC. Gene expression is under the control of the *AOX1* promoter, one of the two promoters for the gene of alcohol oxidase (AOX), a hydrogen peroxide-producing oxidase which metabolize methanol to formaldehyde in the peroxisome of yeast cell. The *AOX1* promoter is repressed during growth of the yeast cell on common carbon sources, e.g. glucose, glycogen, but is highly induced during growth on methanol (Tschopp *et al.*, 1987 b). Crocodile normal TTR and its chimeras, with N-terminus of human TTR and of *Xenopus* TTR, were extracellular expressed with the TTR, itself, secretory signal and secretion

signal of protein α-factor in *Pichia*. Thyroid hormones, both T3 and T4, was studied to determine if these N-terminus changings altered the function, namely the binding affinities to thyroid hormones, of the TTR tetramer. The result clearly demonstrated that both chimeric TTRs have the same binding characteristic as the normal TTR, i.e. bind with T3 better than T4. Correlation of structural analyses and thyroid hormone binding of these proteins could reveal the important role of the N-terminal segment of TTR as one of the functional selection pressure operating in evolution of TTR.

Objectives

1. To generate recombinant chimeric crocodile TTRs having variations in the N-terminal region in order to obtain sufficient amount of the TTRs consisting correct N-terminal sequence as constructed.
2. To determine the Kd values for T3 and T4 of the recombinant chimeric crocodile TTRs and compare to the values of the normal TTR to elucidate the influence of the N-terminal region to the binding affinities to thyroid hormones
3. To prepare the recombinant chimeric crocodile TTRs for structure analysis by X-ray crystallography in future