BEYOND CARRIER PROTEINS

Albumin, steroid hormones and the origin of vertebrates

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Abstract

Albumin, the major serum protein, binds a wide variety of lipophilic compounds including steroids, other lipophilic hormones and various phytochemicals and xenobiotics that bind to receptors for steroids and other lipophilic hormones. Despite albumin's low affinity ($K_d \sim 10^{-4}$ M to 10^{-6} M) for these lipophilic compounds, the high concentration of albumin in serum makes this protein a major carrier of steroids and lipophilic hormones and a regulator of their access to receptors. Albumin also functions as a sink for xenobiotics, diminishing the binding of xenobiotics to hormone receptors and other cellular proteins. This protects animals from endocrine disruption by xenobiotics. We propose that these properties of albumin were important in protochordates and primitive

vertebrates, such as jawless fish, about 600 to 530 million years ago, just before and during the Cambrian period. It is at that time that the ancestral receptors of adrenal and sex steroids – androgens, estrogens, glucocorticoids, mineralocorticoids, and progestins – arose in multicellular animals. Albumin regulated access of steroids to their receptors, as well as protecting animals from endocrine disruptors, such as phytochemicals, fungal chemicals and phenolics, and other chemicals formed at hydrothermal vents by geochemical processes. Thus, animals in which albumin expression was high had a selective advantage in regulating the steroid response and avoiding endocrine disruption by xenobiotics.

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Introduction

The concentration of albumin in human serum is about 45 mg/ml (0.67 mM), making albumin the major protein in serum. Albumin's principal functions are considered to be regulating the osmotic pressure in blood and transporting fatty acids and other lipophilic compounds (Kragh-Hansen 1981, Peters 1985). It has been suggested that albumin is not an essential protein because analbuminemic humans and rats appear to function normally (Boman *et al.* 1976, Nagase *et al.* 1979, Tarnoky 1980). However, analbuminemic humans and rats have 1/1000 normal albumin levels, so they are not truly without albumin, leaving open the possibility that albumin is an essential protein. Also contributing to scepticism about the requirement for albumin is its low affinity for steroids and other lipophilic compounds which bind albumin with an equilibrium dissociation constant (K_d) of 10^{-6} M to 10^{-4} M. This affinity is substantially lower than that of other carrier proteins, such as sex hormone binding globulin and corticosteroid binding globulin, which have K_d s of 10^{-10} M to 10^{-8} M for steroids (Dunn *et al.* 1981).

I have presented an alternative view, proposing that albumin had a role in steroid hormone signaling early in

the evolution of vertebrate albumin, and that this function is retained in modern organisms (Baker 1998). That is, albumin was an ancient carrier protein for steroids, thyroid hormone, retinoids and other lipophilic hormones in primitive vertebrates. In this model, albumin's 'fuzzy recognition' of lipophilic hormones, which is reflected in its low affinity for these compounds, is important in its function. In fact, it provides a selective advantage to its host because it allows albumin to act as a carrier for many different hormones. I also proposed that this fuzzy recognition is important in another function of albumin: protecting lipophilic hormone receptors, as well as enzymes that regulate steroid hormone action (Baker 2001b) and signal transducers such as kinases, from unwanted occupancy by exogenous lipophilic compounds. A current example of this problem is the binding of phytochemicals and synthetic chemicals to the estrogen receptor, which can disrupt reproduction and development in vertebrates.

Here I revisit my earlier evolutionary analysis, using the considerably expanded database of steroid receptor sequences that has accumulated in the last few years. I use this new information to investigate further the relationship between the evolution of albumin, steroid hormone action and the origin of vertebrates.

Figure 1 Compounds that bind to albumin and nuclear receptors.

Adrenal and sex steroid receptors originated about 600 to 530 million years ago

The adrenal and sex steroids – cortisol, aldosterone, estrogen, testosterone, and progesterone (Fig. 1) – have a central role in development, reproduction and homeostasis in humans and other vertebrates. These steroids act through nuclear receptors, a diverse group of transcription factors that also includes receptors for retinoids, thyroid hormone, prostaglandins and fatty acids, as well as receptors without a known ligand, the orphan receptors (Laudet 1997, Owen & Zelent 2000).

Although nuclear receptors are ancient and found in invertebrates, adrenal and sex steroid receptors appear to be found only in vertebrates or their deuterostome ancestors. Support for this hypothesis comes from Escriva *et al.* (1997), who presented compelling evidence that nuclear receptors for adrenal and sex steroids are a ‘recent’ innovation that arose in protochordates and the earliest vertebrates. Phylogenetic analysis of the hormone-binding domain of nuclear receptors supports this hypothesis that steroid receptors arose prior to or during the Cambrian period (Baker 1997, 2001a).

Evolution of the hormone-binding domain of adrenal and sex steroid receptors

Several evolutionary analyses have been reported for adrenal and sex steroid receptors, all showing that they form a separate clade in the nuclear receptor family (Baker 1997, 2001a, Laudet 1997, Owen & Zelent 2000, Thornton 2001). Figure 2 shows an updated phylogeny of the hormone-binding domain of adrenal and sex steroid receptors, using the recently reported sequences for steroid receptors in lamprey (Thornton 2001), a jawless fish that has ancestors in the Cambrian period about 530 million years ago.

As found in other analyses, the estrogen receptor (ER) is in a separate clade from the androgen receptor (AR), progesterone receptor (PR), glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). This clustering is consistent with the properties of these receptors. For example, the AR, PR, GR and MR can bind to the same DNA sequence in the nucleus to regulate gene transcription.

The positions in the phylogenetic tree of the hormone-binding domain on the lamprey GR and PR are puzzling.

Figure 2 Phylogenetic analysis of the hormone binding domain of adrenal and sex steroid receptors. The ClustalW program (Thompson *et al.* 1994) (<http://www.ebi.ac.uk/clustalw/>) with 10 000 bootstrap trials was used to construct a phylogenetic tree of the hormone-binding domain of adrenal and sex steroid receptors, thyroid hormone receptor and retinoid X receptor (RXR). Branch lengths are proportional to the distances between each protein. The bootstrap value for each branch of the tree, which is the number of times this cluster was found in the 10 000 bootstrap trials, is in parentheses.

Instead of lamprey GR and PR each being close to the GR and PR respectively, the hormone-binding domain of lamprey GR and PR cluster together, much like ER α and ER β . The hormone-binding domains on lamprey GR and PR are closest to the MR clade.

Regrettably, a similar analysis of the DNA-binding domain of the sequences in Fig. 2 did not clarify the evolution of the lamprey PR and GR. The analysis showed that the DNA-binding domain of lamprey GR was closest to that of vertebrate GR (data not shown). However, the bootstrap values for the DNA-binding domain of lamprey PR with the vertebrate PR were too low – 3333 out of 10 000 trials – to allow assigning this domain to the vertebrate PR clade.

Thus, at this time, the location of lamprey GR and PR must be considered to be tentative due to the lack of steroid receptor sequences from intermediate organisms such as sharks which arose after lampreys and before bony fish. Indeed, clarification of the location of lamprey GR and PR in the steroid receptor family will come from analysis of the sequence of shark PR and GR and from determining if the MR is present in sharks. This could determine if diversification of steroid hormone action in PR and GR arose initially by divergence in their DNA-binding domains.

Steroid receptors and the origins of vertebrates

Two genome size duplications occurred early in the evolution of vertebrates, before the evolution of fishes with jaws (Sidow 1996, Holland 1999). Each genome size duplication substantially increased the raw material for diversification of protein function and complex regulatory networks that are characteristic of vertebrates. The distances between the branches at the duplications in the ER, AR, PR, GR and MR are short, which indicates diversification by a series of closely spaced gene duplications (on a geological time scale). The chromosomal location of steroid receptors indicates that it is likely that they formed during these genome size duplications. Indeed, the diversification of steroid responses accomplished by the evolution of the ER, AR, PR, GR, and MR probably contributed a selective advantage to ancestral vertebrates during and after the Cambrian explosion.

What was the ancestral steroid receptor?

The phylogenetic tree (Fig. 2) shows that the branch leading to the ER from the ancestral steroid receptor is shorter than the branch leading to the AR, PR, GR and MR, indicating a slower change in the ER sequence since their separation. This suggests that the ER was under functional constraints during this time, which is consistent with the estrogen response being the most ancient of the

adrenal and sex steroid responses. A gene duplication led to separation of the ER from the AR/PR/GR/MR, which recognize 3-keto-steroids.

The identity of the steroid(s) that regulated the action of the primitive ER is not known. It could have been estradiol (Thornton 2001) or a Δ 5-androgen (Baker 2002), both of which bind with high affinity to human ER (Kuiper *et al.* 1997). Or it could have been another steroid or a ligand with a non-steroidal structure, such as a phytochemical.

Carriers for lipophilic hormones in blood

Protochordates and vertebrates have a closed circulatory system in which hormones are synthesized in one organ and transported in the blood to target cells containing a cognate hormone receptor, where the hormone binds to its receptor and regulates the transcription of genes that evoke a characteristic physiological response. Carrier proteins in the blood are important in transporting lipophilic hormones to target cells. These carrier proteins include sex hormone binding globulin (SHBG), which binds estradiol and testosterone, corticosteroid binding globulin (CBG) (Dunn *et al.* 1981, Rosner 1990) and thyroxine binding globulin (TBG). None of the binding globulins SHBG, CBG and TBG is homologous to albumin. However, TBG and CBG are distant homologs which are descended from a protease inhibitor in the serpin family.

When did carrier proteins arise?

Our search of GenBank revealed that only the mammalian sequences of SHBG, CBG and TBG have been determined (data not shown). Albumin has an earlier ancestry; albumin is found in the lamprey (Gray & Doolittle 1992), a cyclostome that arose close to the origins of vertebrates. Cyclostomes and mammals last shared a common ancestor about 450 to 500 million years ago, suggesting that albumin arose either in a protochordate or early in the origins of vertebrates about 600 to 530 million years ago, prior to or during the Cambrian period. Proteins with sequence similarity to albumin are not found in invertebrates, including the complete genome for *Drosophila melanogaster* and *Caenorhabditis elegans*. There is, however, a protein called Endo16 in the sea urchin, which is at the base of the deuterostome line leading to vertebrates, that clearly has a motif of cysteine residues that is characteristic of albumin (Soltysik-Espanola 1994). Endo16 is associated with the plasma membrane. The function of Endo16 is unknown; homologs of Endo16, also with unknown function, are found in mammalian sequences deposited in GenBank. It appears that duplication of Endo16 led to the ancestral albumin. Later duplication of albumin led to alpha-fetoprotein, which binds estrogens in rat and mice (Savu *et al.* 1981).

Thus, it is likely that an ancestral albumin was present during the origins of various ligand-activated nuclear receptors in protochordates and primitive vertebrates. We propose that at that time albumin was the principal carrier for steroids and regulator of steroid access to its receptor, as well as a protector of steroid receptors from occupancy by chemicals formed by plants, fungi, bacteria and at hydrothermal vents by geochemical reactions.

Albumin regulates steroid access to their receptors

Albumin binds a wide variety of hydrophobic ligands including steroids, fatty acids, retinoids, thyroid hormone, prostaglandins and antibiotics (Kragh-Hansen 1981, Peters 1985, Kragh-Hansen *et al.* 1990). As mentioned earlier, the equilibrium dissociation constants (K_{ds}) for steroids are in the 10^{-6} M to 10^{-4} M range. This contrasts with K_{ds} of 10^{-10} M to 10^{-9} M that steroids, retinoids and thyroid hormone have for their nuclear receptors, and with K_{ds} from 10^{-10} to 10^{-8} that steroids and thyroxine have for SHBG, CBG and TBG. However, despite albumin's low affinity for steroids, albumin's high concentration enables it to bind most of the estradiol and a substantial part of testosterone in male and nonpregnant female serum in the presence of SHBG (Dunn *et al.* 1981, Sodergard *et al.* 1982). For example, serum albumin regulates the access of estradiol and its metabolite, estriol, to the estrogen receptor (Anderson *et al.* 1974). This effect depends on the different affinity for estradiol and estriol for albumin (Dunn *et al.* 1981, Kragh-Hansen 1981, Sodergard *et al.* 1982, Peters 1985). Estriol has 1/3 the affinity of estradiol for the estrogen receptor. However, in the presence of albumin, the apparent affinity of estriol is twice that of estradiol for the estrogen receptor because estriol has a lower affinity than estradiol for albumin. Walent and Gorski (1990) also found that albumin could reduce the binding of estradiol to the estrogen receptor.

Albumin regulates access of exogenous chemicals to the estrogen receptor

Animals accumulate lipophilic compounds in their blood when they consume plants, fungi, and bacteria. Some of these compounds are important nutrients. Other compounds can bind to either hormone receptors (Martin *et al.* 1978, Miksicek 1993, Kuiper *et al.* 1997, 1998) or enzymes (Ibrahim & Abul-Hajj 1990, Miller & O'Neill 1990, Adlercreutz *et al.* 1993, de Azevedo *et al.* 1996). Binding of these exogenous compounds to hormone receptors or enzymes (Baker 2001b) can disrupt endocrine responses. Figure 3 shows compounds from plants and a fungus, and synthetic plastics, all of which bind to the ER. Flavonoids also inhibit aromatases (Ibrahim & Abul-Hajj 1990) which convert testosterone to estradiol. Albumin reduces the binding of flavonoids and other lipophilic

compounds to the estrogen receptor (Nagel *et al.* 1998) allowing albumin to regulate access of xenobiotics to a protoestrogen receptor early in the evolution of vertebrates and their protochordate ancestors. Interestingly, rat alpha-fetoprotein, which binds estrogens, also binds phytochemicals (Garreau *et al.* 1991, Baker *et al.* 1998). Flavonoids also bind to SHBG (Martin *et al.* 1996), another indication that the steroid-binding domain on a carrier protein can recognize non-steroidal compounds.

The synthesis of flavonoids and other animal-toxic compounds by plants is an example of coevolutionary interaction between animals and plants (Baker 1995, Ames & Gold 1997, Adlercreutz 1998), in which plants synthesize a chemical that is toxic to animals, animals evolve a defence, and then a new compound is synthesized in plants that will retard its consumption by animals. Animals defend against these toxic compounds by degrading the toxic phytochemical to render it inactive, or by modifying it, for example by conjugation with glucuronic acid, which renders it soluble and suitable for excretion.

Albumin has a role in the detoxification process by retarding the binding of phytochemicals and other xenobiotics (Soto *et al.* 1991, vom Saal *et al.* 1995, Nagel *et al.* 1998) to hormone receptors, hormone carrier proteins and enzymes, which allows the inactivation and excretion of xenobiotics to occur without disruptive endocrine effects.

Fuzzy albumin: a selective advantage in the Cambrian period

The role of albumin as a regulator of access of steroids and other lipophilic compounds to their receptors and as a protector of animals from the disruptive effects of xenobiotic binding to hormone receptors and enzymes would be important in the survival and expansion of vertebrates during and after the Cambrian period from 540 to 520 million years ago (Sidow 1996, Fortey *et al.* 1997). The causes of the Cambrian explosion are not fully understood. Larger animal body sizes during the Cambrian period are thought to depend on increased atmospheric oxygen, which supported the metabolism necessary for growth of larger animals (Canfield & Teske 1996). This larger size requires an increased consumption of plants and small animals, and the need to control the toxic effects of compounds in these foods that could bind to hormone receptors and enzymes. Even now, we ingest more toxic chemicals from plants than synthetic chemicals (Ames & Gold 1997).

Another factor that contributed to the importance of an albumin-like protein during the Cambrian period was the co-evolutionary arms race between plants and animals which increased the exposure of animals to toxic phytochemicals. Animals that had a protein with low selectivity or 'fuzzy recognition' for lipophilic compounds had a selective advantage in avoiding endocrine disruption by

Figure 3 What is an estrogen? Compounds from plants (coumestrol, naringenin, genistein), fungi (zearalenone), synthetic estrogens (diethylstilbestrol, 4-hydroxy-tamoxifen) and plastics (bisphenol A, 4-nonylphenol) bind to the estrogen receptor. This illustrates the diverse sources and structures of compounds that are 'estrogens'.

xenobiotics. A protein with low affinity for lipophilic molecules can control their free concentration if the protein concentration is in excess of the lipophilic molecule's concentration (Silhavy *et al.* 1975). Such a protein needs high aqueous solubility, stability – which can be achieved with disulfide bonds – and it must not be 'expensive' to synthesize; that is, it must not require amino acids such as tryptophan, which are uncommon in food. Albumin is the product of these evolutionary pressures on an ancestor of Endo16. The combination of fuzzy recognition of small molecules with structures consisting of rings or aliphatic chains with different degrees of desaturation (Kragh-Hansen 1981, Peters 1985) and albumin's greater than 500 μM concentration in serum means that albumin will exceed the concentration of many xenobiotics in serum, which will help diminish unwanted binding to receptors and enzymes and promote elimination of xenobiotics.

Chance and the evolution of albumin

It is likely that several proteins had the above attributes of albumin prior to and during the Cambrian period. The 'choice' of albumin may have been a chance mutation that increased its expression in a protochordate, conferring a

selective advantage to an animal in regulating steroid hormone action and/or avoiding endocrine disruption from phytochemicals, fungal chemicals and other xenobiotics. The initial chance choice of albumin for either or both functions in a protochordate set the course for the future function of albumin vertebrate descendants, in which albumin is a high capacity, low affinity binder of lipophilic compounds, maintaining osmotic homeostasis in animal physiology.

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