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The binding of the fluorescent ATP analogue 2′(3′)-trinitrophenyladenosine-5′-triphosphate to rat liver fatty acid-binding protein

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The less polar fluorescent analogue of ATP, 2′(3′)-trinitrophenyl-5′-triphosphate bound to rat liver fatty acid-binding protein with high affinity (K_d 6.3 × 10^{-6} M) and 1:1 molar stoichiometry. This probe bound to the fatty acid binding site of the protein and was displaced by oleic acid and oleoyl CoA. High concentrations of ATP did not cause significant displacement of the fluorescent ATP analogue. Since the anionic part of this molecule is the triphosphate group it is difficult to envisage this group being accommodated at an anion binding site within the non-polar core of this protein as is the case with other fatty acid binding proteins. Therefore it is anticipated that the ligand must bind to liver fatty acid-binding protein with this triphosphate group surface exposed. Caution must be exercised when using the more hydrophobic fluorescent analogue of ATP to investigate the ATP binding properties of proteins.

Rat liver fatty acid-binding protein; ATP; Ligand binding; Fluorescence

1. INTRODUCTION

Fatty acid-binding protein (FABP) from rat liver cytosol is an abundant 14000-M_r monomeric protein that is known to have a high affinity for long chain fatty acids and their CoA esters, as well as a number of non-polar anions [1]. The precise physiological function of this protein remains unclear although it is accepted that the protein must be involved in the transport of fatty acids within the cell [2]. For an intracellular protein to have a role in transport it would be anticipated that mechanisms would exist both for the uptake and release of the transported ligand at different sites within the cell. We have investigated factors that might facilitate binding of labelled FABP to microsomal membranes as the initial event in releasing bound fatty acid to potential fatty acid metabolising systems such as the fatty-acyl CoA synthase. During the course of this work we observed that physiological concentrations of ATP stimulated the binding of liver FABP to liver microsomal membranes (Sheridan and Wilton, unpublished observations). In an attempt to further define this phenomenon we investigated the binding of the fluorescent ATP analogue TNP-ATP to FABP. The results highlight the ability of TNP-ATP but not ATP to bind within the hydrophobic core of the protein as a result of the increased hydrophobicity of the adenine-ribose part of the ATP analogue.

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Abbreviations: FABP, fatty acid-binding protein, TNP-ATP, 2′(3′)-trinitrophenyladenosine-5′-triphosphate; DAUDA, 11-(dansylamino)undecanoic acid.

2. EXPERIMENTAL

2.1. *Materials*

TNP-ATP and DAUDA were obtained from Molecular Probes, Junction City, OR, USA. Rat liver FABP was prepared according to published methods [3].

2.2. *Methods*

Fluorescence measurements were performed in 0.01 M potassium phosphate buffer pH 7.4 (2 ml) containing the appropriate ligands. Up to 0.05 ml of the added ligand was titrated into the cuvette and the fluorescence measurement corrected for dilution. Oleic acid was added in methanol and a correction for this solvent was also performed. Titrations involving TNP-ATP were corrected for inner filter effects. Excitation was at 408 nm and maximum fluorescence was obtained by scanning between 500 and 580 nm.

3. RESULTS

3.1. *Binding of TNP-ATP to FABP*

The fluorescent ATP analogue TNP-ATP has been used to study ATPases and was initially shown to bind to hydrophobic areas of heavy meromyosin ATPase with an accompanying increase in fluorescence intensity and decrease in wavelength of maximum emission [4].

When TNP-ATP fluorescence spectrum in buffer was

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compared with that in the presence of an equimolar concentration of FABP a shift in wavelength maximum and increase in fluorescence intensity was observed consistent with the probe binding within a non-polar environment (Table I). The wavelength of emission maximum (536 nm) is equivalent to the probe being dissolved in excess of 70% (v/v) ethanol [5]. This non-polar location would be consistent with the chromophore being buried within the hydrophobic core of liver FABP.

3.2. *The stoichiometry of binding TNP-ATP to FABP*

Fixed concentrations of rat liver FABP were titrated with TNP-ATP and the resulting binding curve is presented as a Scatchard plot (Fig. 1). In order to quantify the amount of bound ligand the fluorescence intensity of a known concentration of TNP-ATP was determined by adding increasing amounts of FABP. When the reciprocal of fluorescence intensity was plotted against the reciprocal of FABP concentration a linear plot was obtained from which was calculated the fluorescence intensity for when the probes was completely bound to protein.

The Scatchard plot identified one high affinity site on the protein with a K_d of 6.3×10^{-6} M and the possibility of further non-specific binding sites which could not be determined due to excessive fluorescence quenching by the probe.

A K_d of 6.3 μM for TNP-ATP is about one order of magnitude high than value reported for fatty acids binding to this FABP and more consistent with values reported for complex non-polar anions such as oestrone sulphate [6] and various bile acids [7].

3.3. *Displacement of FABP-bound TNP-ATP by long chain fatty acids fatty acyl CoAs and ATP*

In order to gain information as to the precise nature of the TNP-ATP binding site on FABP, the ability of different types of ligands to displace bound TNP-ATP was investigated. In each case, after recording the fluorescence enhancement achieved by mixing 10 nmol of FABP and 10 nmol of TNP-ATP, the competing ligand was titrated into the mixture and the fall in fluorescence was recorded. Over 90% displacement was achieved by the addition of 100 nmol of palmitoyl CoA or oleoyl CoA while 10 nmol of these ligands produced about 50% displacement. Oleic acid at 10 nmol produced about 70% displacement consistent with the fact that long chain fatty acids have a higher affinity for FABP than does TNP-ATP and that fatty acyl CoAs bind with lower affinity than the corresponding fatty acids. The very low solubility of oleic acid prevented the use of higher concentrations of this ligand. These results strongly suggest that the TNP-ATP was binding within the protein at the non-polar anion binding site.

When the ability of ATP, ADP, AMP and CoA to displace TNP-ATP was examined no significant loss of fluorescence was observed when up to 500 nmol of these ligands were added. This result is consistent with the observation that ATP was unable to displace DAUDA from FABP (see below) indicating that ATP does not bind at the normal non-polar anion binding site within core of the protein.

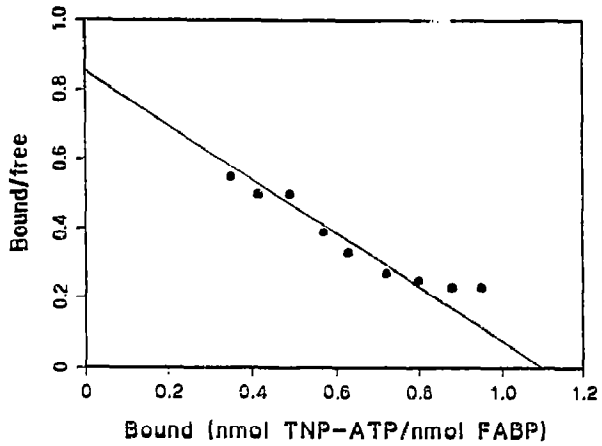

Fig. 1. Scatchard analysis of TNP-ATP binding to FABP. TNP-ATP, 2.5 to 50 nmol, was titrated into 10 nmol of FABP in 2 ml of potassium phosphate buffer, pH 7.4, at room temperature.

3.4. *Displacement of FABP-bound DAUDA by ATP*

The experiments described above demonstrated a direct interaction of ATP analogue with liver FABP. The primary ligand binding domain of FABPs is now accepted to be within the β-clam structure that was initially identified as the basic structure for intestinal-FABP [8] and the P2 myelin protein [9] while this basic structure has more recently been confirmed for adipocyte lipid binding protein [10]. In the case of liver-FABP the crystal structure is not available however NMR data suggests a very different orientation of ligand binding than that now seen for the other binding proteins [11]. In particular it has been concluded that the carboxylate anion is surface exposed and this could explain the ability of liver FABP to bind a wide variety

Table I

The fluorescence characteristics of TNP-ATP bound to rat liver FABP

Experiment	Maximum emission wavelength (nm)	Fluorescence intensity (arbitrary units)	Number of assays
Buffer + TNP-ATP	574 ± 1	2.2 ± 0.9	13
Buffer + +TNP-ATP +FABP	536 ± 1	8.1 ± 2.5	13

Measurements were performed in 10 mM potassium phosphate buffer pH 7.4 (2 ml) containing 10 nmol of TNP-ATP or 10 nmol of TNP-ATP + 10 nmol of rat liver FABP. Excitation was at 408 nm.

on anionic non-polar ligands. Such ligands would have the nonpolar part of the molecule located in the core of the β-clam structure and if TNP-ATP was a faithful analogue of ATP then ATP should also show binding within the core of the protein. Interaction of ATP with this internal domain should be detectable using displacement of the fluorescent fatty acid probe DAUDA [12] and this probe is displaced by established ligands for liver-FABP. However no significant displacement of DAUDA by ATP was observed using concentrations of ATP up to 5 mM (data not shown).

4. DISCUSSION

Attempts to mimic a possible interaction of ATP with FABP using the fluorescent ATP derivative TNP-ATP identified the capacity of FABP to bind this less-polar ATP analogue with high affinity and with a 1:1 molar stoichiometry. Displacement studies using oleic acid and oleoyl CoA confirmed that TNP-ATP was binding at the normal non-polar ligand binding site on FABP. ATP at high concentrations was unable to cause significant displacement of either TNP-ATP or the fluorescent fatty acid probe, DAUDA. Thus the ATP analogue TNP-ATP, being less polar, tends to bind to non-polar binding domains of proteins to an extent not seen with ATP. Hence caution must be used in interpreting fluorescent data based on the assumption that the probe is mimicing the behaviour of ATP.

The capacity of TNP-ATP to bind with high affinity to liver FABP is a dramatic example of the broad specificity of this binding protein and its capacity to bind a range of non-polar ligands having a variety of polar anionic head groups. This broad specificy is most readily explained if the head group was surface exposed and not accommodated within the core of the protein as indicated by NMR studies [13]. It is known that other fatty acid binding proteins for which a crystal structure is available are selective for fatty acids and the carboxyl is buried within the core and bonds to an arginine residue [8–10]. We have mutated the internal arginine residue in liver FABP (Arg-122) with minimal effect on ligand binding thus supporting a different ligand orientation in this protein (A. Thumser, C. Evans, A.F. Worrall and D.C. Wilton, manuscript in preparation). The binding of TNP-ATP represents an extreme example of a non-polar ligand with a highly charged and bulky anionic group. It is hard to envisage this ligand binding other than with the triphosphate group surface exposed whereas the adenine ribose moiety where the ribose is modified with the fluorescent trinitrophenyl group is located within the non-polar core of the protein.

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