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Binding of ATP to uncoupling protein of brown fat mitochondria as studied by means of spin-labeled ATP derivatives*

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ATP derivatives spin-labeled (SL) at C8, N^6, C2′ or C3′ were employed in binding studies with the uncoupling protein of brown fat mitochondria. Substitution of the ribose strongly impaired binding, whereas labeling of the adenine moiety allowed for tight and functional complex formation. Detailed binding studies with C8-SL-ATP confirmed the known pH and Mg^{2+} dependence with a stoichiometry of one C8-SL-ATP bound per 66 kDa dimer. Corresponding studies of the uncoupling protein after modification with *N*-ethylmaleimide or diazobenzene-4-sulfonic acid revealed distinct differences in their effects on nucleotide binding and gating.

Uncoupling protein; Chemical modification; Brown fat mitochondria; ATP binding site; Spin-labeled ATP

1. INTRODUCTION

The uncoupling protein (UCP) utilizes the proton motive force of the inner mitochondrial membrane for thermogenesis. The molecular mechanism of the H^+ translocation by UCP is of particular interest because of its regulation, i.e. gating by purine nucleotides [1–5]. Functional UCP has been reported to be a dimer of two 33 kDa subunits, however, bearing a single binding site for purine nucleotides only [4]. The same stoichiometry was found in the case of the ATP/ADP carrier [6]. The binding of purine nucleotides to UCP results in closing of both known transport pathways – the H^+ channel [7] and the monovalent anion channel [8]. The nucleotide binding site exhibits a rather broad specificity for the purine ring including adenines, guanines, inosines, xanthines, and even 8-azido or 8-bromo derivatives, with high affinities for their di- and triphosphates [9]. Adenosine 5′-β,γ-methylene-triphosphate (AMP-PCP), adenosine 5′-β,γ-imidotriphosphate (AMP-PNP) and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) bind nearly as well [1], but adenosine 5′-α,β-methylenetriphosphate (AMPCPP) and monophosphates exhibit low affinity. Pyrimidine nucleotides do not exhibit gating [10]. Gating is also completely prevented in UCP modified by NEM or DTNB [11]; incorporation of DABS was reported to prevent high affinity binding with the inhibitory curves being shifted to very high GDP concentrations [5].

Spin-labeled nucleotides have found a wide application in the study of enzyme mechanisms [12]. Spin-labeled derivatives of ATP labeled at N^6 or C8 of the adenine ring [13] or at the C3′ position of the ribose [14] (Fig. 1) have been successfully utilized in the study of F_1-ATPase [15] and Ca^{2+}-ATPase [13,16]. ESR binding studies employing spin-labeled ligands provide the distinct advantage of a fast and sensitive direct method by measuring the concentration-dependent signal amplitude of the freely tumbling species. Thus, data are not based on an ambiguous end value as in most other spectroscopic techniques. In addition, conformational changes of the protein are often revealed by changes in the ESR line shape [17]. More recently, even intramolecular distances between spin-labeled nucleotides bound to distinct sites within the protein have been determined by ESR. Spin-spin interaction between these ligands resulted in complex spectra due to dipolar splitting of the lines [18–20]. In this paper we describe the first application of spin-labeled ATP derivatives for the investigation of UCP.

2. MATERIALS AND METHODS

C8-SL-ATP and N^6-SL-ATP were synthesized according to Glöggler et al. [13], C8-SL-AMP according to Wenzel and Trommer and

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Abbreviations: UCP, uncoupling protein; C8-SL preceding ATP or AMP refers to their 8-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl)amino derivatives; N^6-SL preceding ATP or AMP refers to their N^6-(2,2,6,6-tetramethylpiperidin-4-yl-1-oxyl) derivatives; C2′,3′-SL preceding ATP refers to the equilibrium mixture of its C2′- and C3′-(2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid esters); UCP, uncoupling protein from brown adipose tissue; NEM, *N*-ethylmaleimide; DABS, diazobenzene-4-sulfonic acid; HEPES, *N*-(2-hydroxyethyl) 1-piperazineethanesulfonic acid; EGTA, ethylenebis (oxyethylenenitrilo)tetraacetate; MES, 2-morpholinoethanesulfonic acid

as modified by Deparade et al. [21,22], N^6-SL-AMP according to Trommer et al. [23] and C2',3'-SL-ATP according to Vogel-Claude et al. [15].

ESR spectra were recorded with a Bruker ESP 300 spectrometer operating in the X-band mode at 15 mW microwave power and a modulation amplitude of 1.0 G. Measurements were performed at 25°C in a total volume of 60 μl of 300 mM sucrose, 50 mM HEPES, 80 mM KCl, 80 μM EGTA, pH 6.3–7.4. The titration technique employed is described in [24]. The fraction of unbound SL-ATP was determined from the amplitude of the high field line on a diagram of amplitude versus concentration resulting from a blank titration without protein. The concentrations are given in the legends to Figs. 4 and 5. Determination of dissociation constants for binding and the number of nucleotide binding sites per protein were evaluated according to Scatchard [25] and by non-linear regression analysis to theoretical binding curves by an iterative procedure [26].

Brown adipose tissue mitochondria were prepared by a standard procedure from Syrian hamsters adapted to cold (5°C) for at least three weeks. Protein concentration was determined according to Lowry [27] using BSA as a standard. UCP was prepared from a Triton X-100 extract of either intact or modified brown adipose tissue mitochondria according to Klingenberg [4]. Details of the chemical modification of mitochondria by NEM and DABS have been described previously in [5,11]. Before isolation, functional assays were performed to verify the phenomena as described in [5,11]. H^+ transport and Cl^--transport were measured as valinomycin-induced passive H^+ extrusion or swelling of mitochondria in KCl as described previously [28,29]. Cl^--dependent swelling was measured in 2 mM tetraethylammonium MES containing 54 mM KCl, 1 μM rotenone, 0.1 μM antimycin and 0.6 μM oligomycin.

3. RESULTS AND DISCUSSION

3.1. *Characteristics of C8-SL-ATP binding to the native uncoupling protein*

First, we have performed comparative binding studies of UCP with ATP derivatives spin-labeled at different positions, i.e. C8-SL-ATP, N^6-SL-ATP and C2',3'-SL-ATP (Fig. 1). Binding of C8-SL-ATP and N^6-SL-ATP was comparable, with the C8 derivative being slightly more effective. The ESR spectrum (Fig. 2) exhibits components in the low field and high field region typical of a highly immobilized species [17]. Binding is site-specific as was shown by the addition of unlabeled ATP and concomitant liberation of bound C8-SL-ATP (Fig. 2C). The C2',3' derivative, on the other hand, did not bind under comparable conditions (up to 1 mM of the analog at 100 μM of the UCP). A change of 2% in the signal amplitude of freely tumbling C2',3'-SL-ATP would have been well above the error limits. This is in good agreement with earlier studies showing that substituents at C3' of the ribose moiety prevent inhibition of transport by purine nucleotides [30]. Likewise, the spin-labeled mononucleotides, C8-SL-AMP and N^6-SL-AMP did not exhibit noticeable binding.

The crucial question inherent in the application of any reporter group is the extent to which this group, e.g. the spin label, causes perturbations of the protein and thus alters its normal behavior. That indeed, C8-SL-ATP represents ATP rather well was shown by its effect on the inhibition of Cl^--uptake (swelling) in

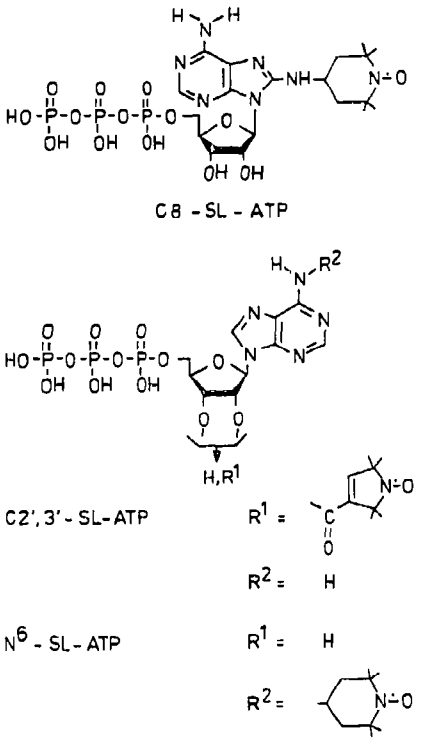

Fig. 1. Structural formulas of C8-SL-ATP, N^6-SL-ATP and C2',3'-SL-ATP. The brackets in the latter indicate an equilibrium mixture of 2'- and 3'-esters.

brown fat mitochondria. As shown in Fig. 3, K_i for inhibition was about 9.6 μM at pH 6.8 as compared to 4.6 μM for ATP itself [28]. It should be noted, however, that the apparent affinity of C8-SL-ATP (and of ATP) to UCP is higher (K_i is lower) in intact mitochondria than to isolated UCP. The reason for that could be the excessive amount of Triton X-100 or residual lipids in our *crude* preparation of UCP.

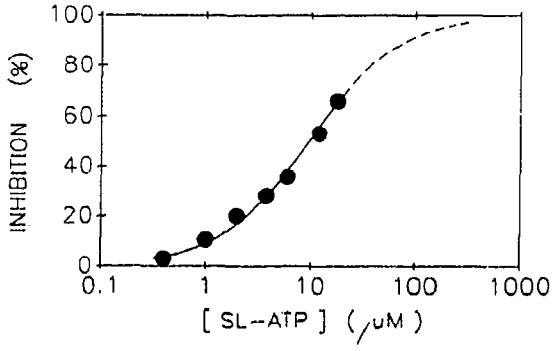

Fig. 2. C8-SL-ATP dose/response for the inhibition of Cl^- transport by UCP as measured by swelling of brown adipose mitochondria (62 μg/ml) in 54 mM KCl at pH 6.8. After 12 s of incubation of aliquots of C8-SL-ATP with mitochondria, transport was induced by 1 μM valinomycin. A line was drawn according to the Hill equation where the intercept and Hill coefficient were taken from the linearized Hill plot constructed from the data. The Hill coefficient was close to 1 (1.015) as is the case with ATP itself, yielding K_i = 9.6 μM.

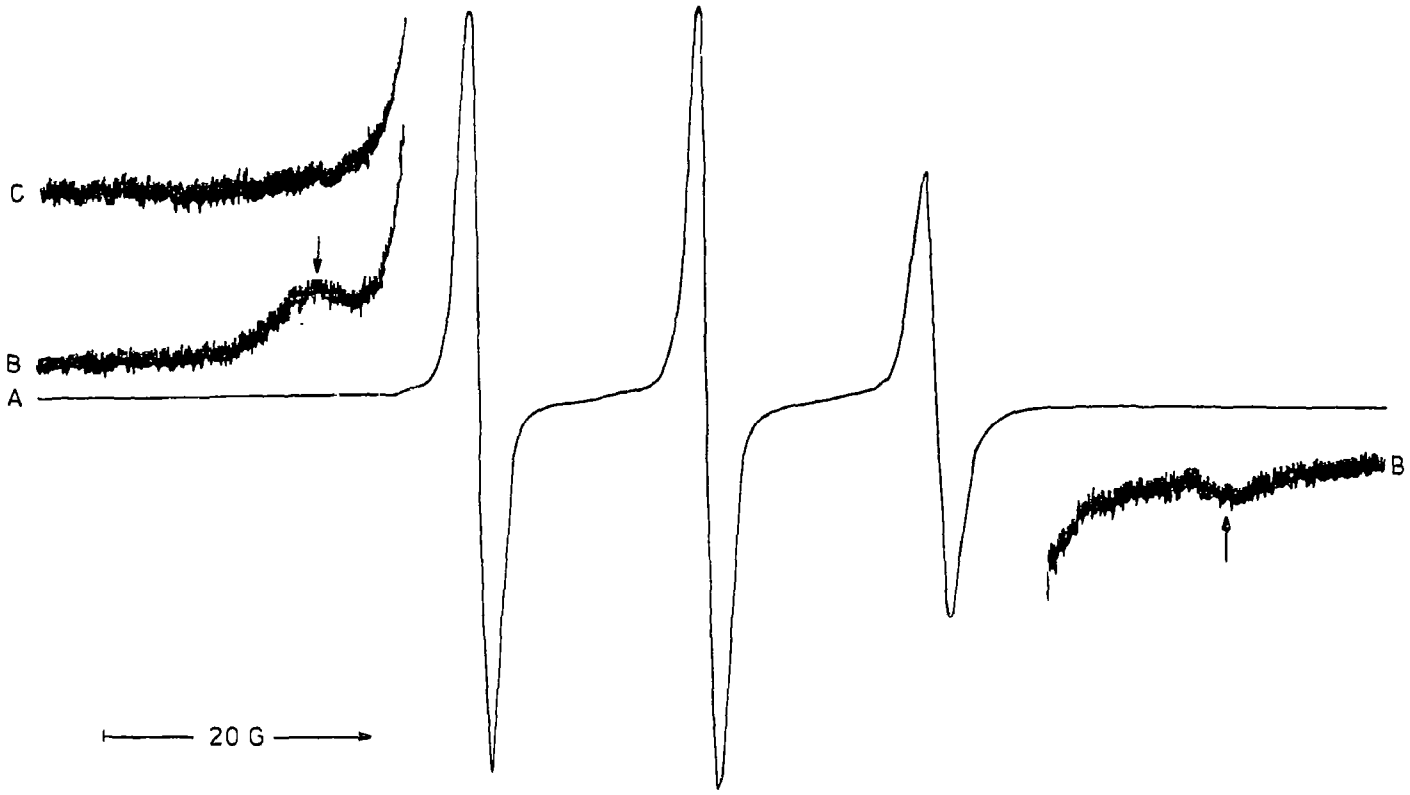

Fig. 3. (A) ESR spectrum of 41 μM C8-SL-ATP in complex with 72 μM UCP at pH 7.0 and (B) with high field and low field regions at 175-fold gain. The signals from the immobilized species are indicated by arrows. (C) Same spectrum after addition of 385 μM ATP.

Detailed binding data which C8-SL-ATP as obtained by ESR titrations revealed a single binding site per UCP dimer of M_r = 66 kDa when evaluated according to both Scatchard [25] as well as non-linear regression analyses to theoretical binding curves by an iterative procedure [26], as shown in Fig. 4. Hence, the ESR titrations confirm earlier findings [4,31]. Presently, we cannot distinguish whether both subunits are essential for the formation of one binding site, or whether one site is simply not accessible, either in the Triton X-100 micelles or in intact mitochondria [31].

The dissociation constant of C8-SL-ATP was found

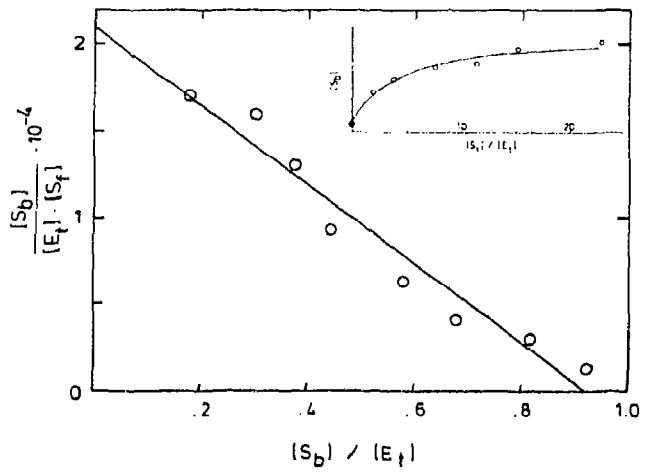

Fig. 4. Scatchard plot of ESR titration data for 48-474 μM C8-SL-ATP binding to 83-101 μM UCP at pH 6.8. The protein concentration was based on the dimer molecular weight of 66 kDa yielding K_d = 50 μM. The inset shows the same data plotted according to a non-linear regression analysis (K_d = 69 μM) [26].

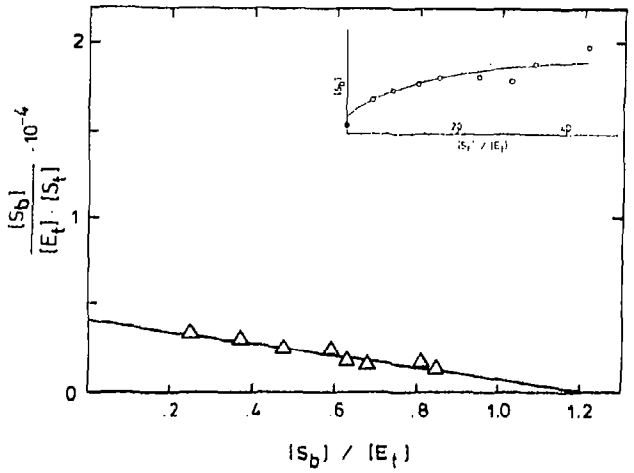

Fig. 5. Scatchard plot of ESR titration data for 96-676 μM C8-SL-ATP binding to 61-80 μM NEM-modified UCP at pH 6.8. The protein concentration was based on the dimer molecular weight of 66 kDa yielding K_d = 300 μM. The inset shows the same data plotted according to a non-linear regression analysis (K_d = 270 μM [26].

to be strongly pH dependent, varying from about 30 μM at pH 6.3 to 300 μM at pH 7.5. The titration at pH 6.8 as shown in Fig. 4 yielded 50–69 μM depending on the method of evaluation. A similar change in the affinity for ATP as determined by a microdialysis technique was described by Klingenberg [1]. The K_d was 0.5 μM at pH 6.0 and increased to 32 μM at pH 7.5. Binding of C8-SL-ATP was also Mg^{2+}-dependent decreasing more than 2-fold in the presence of 10 mM Mg^{2+} to K_d = 135 μM (pH 6.8).

3.2. *C8-SL-ATP binding to chemically modified uncoupling protein*

Earlier studies by chemical modification with NEM and DABS led to quite different changes with respect to the properties of UCP [5,11]. Modification of a thiol group by NEM somewhat altered binding of GDP and prevented gating completely [11]. DABS is rather non-specific with respect to amino acid residues leading to both stable and unstable products [32]. Transport by DABS-modified UCP could still be inhibited by GDP, however, at *about 10-fold* the concentration required for the native protein [5]. Binding studies employing C8-SL-ATP partially confirmed these results. Modification with NEM reduced the affinity of UCP for C8-SL-ATP 6-fold to 300 μM at pH 6.8 as shown in Fig. 5. Non-linear regression analysis by an iterative procedure [26] yielded K_d = 270 μM. The fit, however, was considerably better when the binding curve was calculated for independent, but different sites with K_{d-1} = 86 and K_{d-2} = 360 μM (not shown). The stoichiometry again was close to one C8-SL-ATP molecule bound per dimer as had been the case with native UCP. With DABS-modified UCP, on the other hand, binding of up to 1 mM C8-SL-ATP, if any, was too weak to be observed by ESR.

The intruiging question as to how binding of the nucleotides is related to gating of the proton and/or Cl^- channel cannot be answered as yet. However, with the additional information inherent in the line shape of bound spin-labeled nucleotides, corresponding conformational changes can now be studied in more detail. Work employing covalently bonded photoaffinity spin-labeled ATP derivatives [33] is presently being carried out in our laboratory.

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