Volume 256, number 1,2, 101–105 FEB 07680 October 1989

Arrestin of bovine photoreceptors reveals strong ATP binding

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Received 14 July 1989

The soluble protein arrestin (also named 48K-protein or retinal-S-antigen) is involved in controlling light-dependent transducin and cGMP phosphodiesterase activity in retinal rods. It is also known for its ability to induce autoimmune reactions in the eye causing the eye disease uveitis. We report here a rapid binding of ATP to arrestin with $K_A = 2 \times 10^{21}$ $(l/mol)^3$ and a coordination number $n = 3$. This ATP binding to arrestin supports the notion of a nucleotide exchange which initiates the rapid inhibitory action of this enzyme during the primary step of vertebrate phototransduction.

Rhodopsin; Arrestin; Transducin; ATP binding; ADP/ATP exchange

1. INTRODUCTION

In the vertebrate photoreceptor the signal transduction mechanism is initiated by the light induced formation of a metastable state of the photopigment rhodopsin (37 kDa). From this intermediate state, the meta II rhodopsin, the light activation is rapidly transferred to channel proteins in the envelope membrane which control the ion currents of the receptor. This phototransduction process is mediated by the combined action of 4 receptor proteins: a nucleotide binding protein, transducin (79 kDa), acts as a diffusible transmitter giving alternatively contact between the light-activated meta II rhodopsin and inhibited phosphodiesterase (PDE) molecules [1]. In this step the PDE is activated to hydrolyze cyclic guanosine monophosphate (cGMP) which is supposed to keep ion channels open in the receptor plasma membrane [2]. The transducin process has a high chemical amplification yielding 100–500 activated PDE per bleached rhodopsin [2]. The reaction cascade is finally stopped by the action of a third receptor protein, arrestin (45 kDa), which binds to the meta II rhodopsin. Kühn et al. showed that an effective arrestin binding that suppresses a further PDE activation could not be obtained before bleached rhodopsin is phosphorylated by the activity of a fourth receptor protein, rhodopsin kinase (68 kDa). The phosphorylation takes place at the protein chain which contains the C-terminal end [3–5]. At first sight, this would suggest a binding of arrestin to these phosphate groups at the end chain, known as regulatory unit [6]. This view might further be supported by the recent finding of Yamaki et al. that in the amino acid sequence of arrestin one has three specific binding pockets for phosphate groups [7]. But there is other evidence that arrestin does not necessarily bind this way: suppose the phosphate groups of the activated rhodopsin make contact indeed with the binding pockets of arrestin. Then one would expect that a maximum of 3 of them would be sufficient for a successful binding. The transducin activation of bleached rhodopsin, however, is not blocked by arrestin before more than 6 sites at the regulatory unit are being phosphorylated [8,9]. As a matter of fact, arrestin is found to bind also to the meta II rhodopsin state prior to its phosphorylation. This

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Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; DTT, dithiothreitol; cGMP, cyclic guanosine monophosphate; PAGE, polyacrylamide gel electrophoresis; PDE, phosphodiesterase; ros, rod outer segment; SDS, sodium dodecyl sulphate

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

binding is totally blocked by ATP [10]. These results suggest a different type of binding to occur at the phosphate binding sites of arrestin. Moreover, the sequence analysis studies done by Shinohara et al. show certain similarities between arrestin and the T_α-subunit of transducin especially with regard to a possible binding of nucleotides [11,12].

Actually, this communication gives direct evidence that 3 ATP molecules can bind strongly to arrestin and that this binding is influenced by ADP. This finding gives new insight in the possible role of arrestin as an ADP/ATP-exchange protein and thus a counterpart of transducin in the light regulation of the phototransduction process. This view of arrestin interaction in the phototransduction process is generally applicable whether transducin is regarded as activator of phosphodiesterase activity – as commonly agreed to above – or as a direct blocker of cGMP-opened ion channels – as recently suggested on the basis of new electrophysiological findings [13].

2. MATERIALS AND METHODS

2.1. *Preparation of arrestin*

To obtain highly active arrestin for binding studies, in principle the method described by Kühn [14] and Pfister [15] was used with a special modification concerning the preparation of rod outer segments (ros) to enhance the protein yield: bovine eyes were gathered at the slaughterhouse and kept warm near body temperature until the retinae could be removed 2–3 h after enucleation of the eye bulbs.

The retinae were isolated from the open eye cub and stored in Ringer's solution [16]. Different from the normal procedure, however, under continuous stirring the retinal tissues were illuminated in Ringer's solution for 20 min by the light of a 150 W incandescent lamp filtered by an orange glass filter (Schott, Mainz, FRG, OG 590; 1 mm). After illumination the ros preparation followed the normal procedure [16]. The further preparation of arrestin from light adapted retinal rods was done by the known method cited above [14,15].

A quantitative protein assay was achieved by a method described by Bradford [17]. The protein was determined using SDS-PAGE chromatography [18]. Employing the light adaptation step the yield in arrestin could be enhanced to 7–8% of the original rhodopsin contents as compared to 1–3% without light adaptation [10]. The arrestin obtained by this procedure showed on the average a 75% activity with respect to ATP-binding (see below).

2.2. *Preparation of '100 mM' protein extract*

A second protein extract was obtained from bovine outer segment suspensions which besides arrestin contained rhodopsin kinase, too. For this purpose, a ros-suspension was homogenized by ultrasonification (B12, Branson, Danbury, CT, USA) in a KCl-Tris-buffer (100 mM KCl, 10 mM Tris-HCl, 3 mM $MgCl_2$, 1 mM DTT, pH 7.4) and then centrifuged for 15 min at 45000 × *g* (RC5B, rotor SS-34, Sorvall Inc., Newton, CT, USA). The supernatant solution is the '100 mM' extract which contains the two proteins [10].

2.3. *Measurement of ATP binding*

The binding of ATP to arrestin was measured in a luminometer (1250, LKB, Bromma, Sweden) using the standard buffer and the ATP-monitoring reagent of LKB employing the luciferin/luciferase system as described by Chapelle [19] and Thore [20].

In preliminary, qualitative experiments a certain amount of ATP was given to the test buffer solution first and the corresponding luminescence level measured. Thereafter a small volume (5–10 μl) of the protein-solution – 100 mM extract or arrestin – was added and the decreasing level of luminescence measured. For performing quantitative binding studies the calibration of luminescence levels and the measurement of ATP consumption could readily be done in the luminometer during the same set of experiments. In each case, a series of equal volume aliquots of ATP solution ($l = 1 \ldots N$, $N = 5 \ldots 10$, Δv = 5–10 μl, c_{ATP}: 1×10^{-6} M) was given to the luminometer solution (250 μl) one in addition to the previously added arrestin-extract (approx. 50 pmol), the other one, for calibration, to the pure buffer solution. From these two sets of luminescence levels (cf. fig.3) the amount of free and bound ATP was determined by the height and the difference of corresponding levels at equal number *l*.

3. RESULTS

From the chromatographic SDS-PAGE plots shown in fig.1 it is evident that the arrestin extract from a bovine rod outer segment preparation contains this protein (45 kDa) nearly in its pure form. The other 100 mM protein extract, however, includes both arrestin (45 kDa) and rhodopsin kinase (68 kDa) as well. The rapid ATP binding to arrestin is illustrated in fig.2. In fig.2A the luminescence level is shown which is measured after the addition of ATP by which a concentration of 1 nM is obtained. This luminescence level is decreased within the luminometer time resolution (<0.2 s) as soon as the arrestin-extract is added. In fig.2B the ATP-consumption is measured which is caused by the addition of the 100 mM-protein extract. At first a rapid ATP binding is observed – shown by the negative jump in luminescence level – followed by a further linear decrease of luminescence. Obviously, this protein extract effects both the rapid ATP binding by arrestin and the enzymatic action of the rhodopsin kinase in consuming ATP as well.

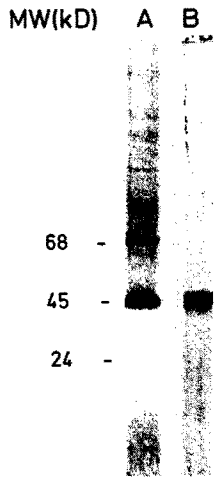

Fig.1. SDS-PAGE gels from 100 mM-extract (A) and pure arrestin-extract (B).

These first qualitative results gave strong evidence that a binding of ATP to arrestin exists at the conditions of these experiments. To study the binding properties the same luminescence method was used. Fig.3 shows a series of luminescence levels measured when equal amounts of ATP are successively given to the luminometer buffer solution. These levels are always lower if arrestin has previously been added (fig.3A) than the corresponding calibration levels (fig.3B) where arrestin has been omitted. The concentration values of free and bound ATP are readily calculated from the corresponding levels.

A plot of bound ATP vs the free ATP on a linear concentration scale (see fig.4) yields the saturation value $c_{A,m}$ of maximum binding of ATP

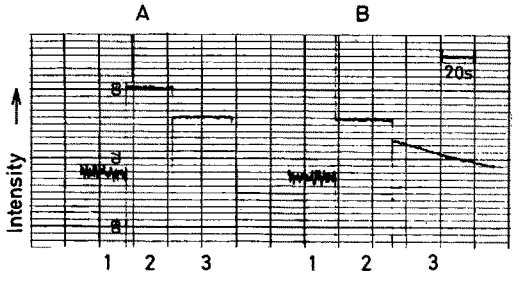

Fig.2. Rapid ATP binding of arrestin (Luminometer 1250, LKB). (A) 1, standard; 2, ATP level (10^{-9} mol); 3, 10 μl arrestin, conc.: 6×10^{-7} M. (B) 1, standard; 2, ATP level (10^{-10} mol); 3, 5 μl 100 mM-extract, conc.: 1.5×10^{-6} M.

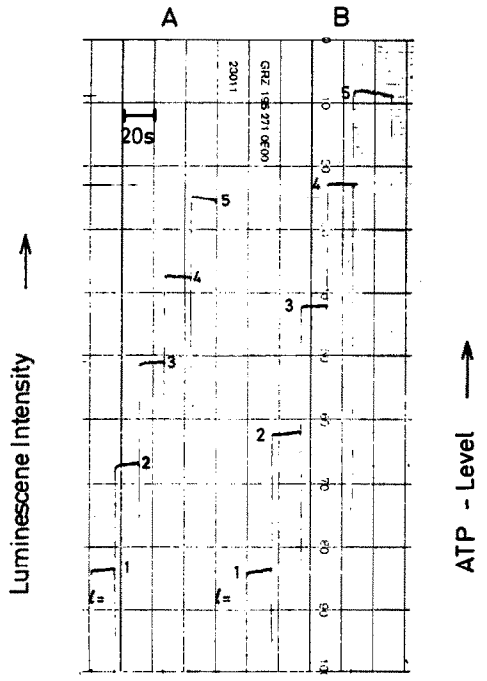

Fig.3. The luminescence of a sample with (A) and without (B) arrestin is measured. Equal volumes (Δv = 8 μl, l = 1...5) of ATP-solution, conc.: 1×10^{-6} M are added. From the difference in corresponding intensity levels (equal number l) between A (free ATP, c_A) and B (total ATP) the amount of bound ATP (Δc_A) is determined.

(A) to arrestin. In case of a stoichiometric binding of ATP (A) to arrestin (S) according to:

$$S + nA \overset{K}{\rightleftharpoons} S \cdot A_n \qquad (1)$$

(K = association equilibrium constant, n = maximal number of ATP-molecules bound to arrestin) the relation for the bound (Δc_A) vs free ATP concentration (c_A) should be:

$$\theta = \frac{\Delta c_A}{c_{A,m}} = \frac{K c_A^n}{1 + K c_A^n} \qquad (2)$$

A numerical fit to this equation (see solid line in fig.4A) as well as separate Hill-plot (fig.4B) according to:

$$\ln \frac{\phi}{1-\phi} = \ln K/c_o^n + n \ln(c_A/c_o) \qquad (3)$$

(where c_o = standard concentration, e.g. nM), yields $n = 3 \pm 0.3$ and $K = (2 \pm 0.3) \times 10^{21}$

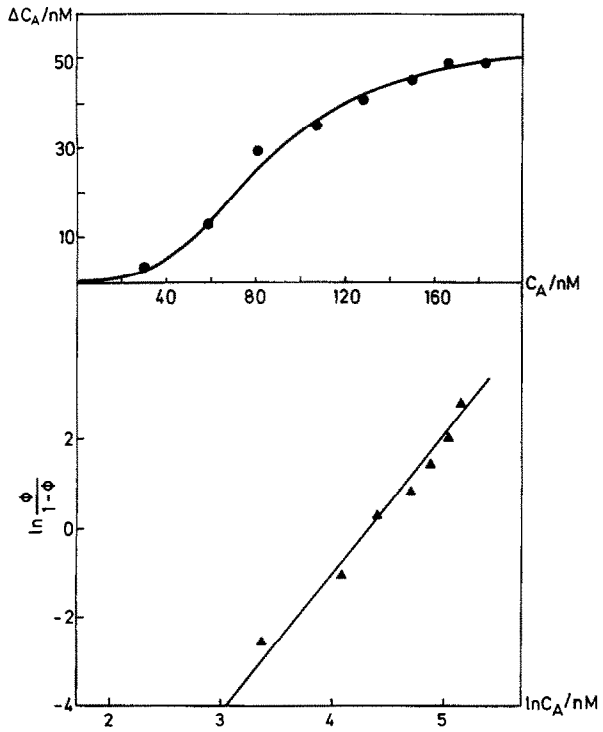

Fig.4. Plot of bound ATP (Δc_A) versus free ATP (c_A). One measurement of five. (Upper part) Linear plot, sigmoidal binding curve; (lower part) logarithmic Hill plot, slope shows coordination number n = 3 (2.98).

(l/mol)^3. These data could be obtained as mean value from at least 5 separate measurements at different preparations and slightly varied procedures.

According to eqn 2 the effective protein concentration c_s^o for ATP binding can be given by the maximum binding capacity $c_{A,m}/n = c_s^o$. On the other hand, at the experiment shown in fig.4A the protein concentration measured by the Bradford method is c_p = 530 nM. A comparison with the experimental value for maximum binding $c_{A,m}$ = (50 ± 5) nM (asymptote in fig.4A) results in an efficiency $\gamma = c_s^o/c_p = 0.95 \pm 0.2$ in the activity of arrestin for ATP binding. In the average overall experiments in this binding study the arrestin preparation has an efficiency $\gamma = 0.75 \pm 0.2$ for stoichiometric binding of ATP.

In additional still preliminary experiments ADP was given also to the test solution: contrary to expectation ADP did not inhibit the binding of ATP to arrestin but seemed rather to promote it. The conclusion is that ADP does not bind directly to arrestin at least not at the same binding sites.

4. DISCUSSION

The binding studies presented in this short communication prove that ATP binds to arrestin with a coordination number of 3 and a relatively high association constant. This finding was supported by the sequence analysis of arrestin published by Yamaki et al. [7] just after the first results of the ATP-binding study were obtained: the amino acid sequence of arrestin shows 3 binding sites for phosphate groups but no specific nucleotide binding areas. On average, each of these phosphate 'pockets' have positive charges in its close vicinity (see fig.5). This finding suggests, that during the ATP binding to arrestin the outer phosphate groups of the negatively charged ATP interact with the positively charged phosphate binding sites. The absolute value of the association constant indicates that the ATP binding to arrestin occurs already at low ATP concentrations. Half-saturation for binding is found at 80 nM of free ATP.

The predominant question to be discussed is whether this particular ATP binding has significance for the signal transmission in the vertebrate photoreceptor. The answer is suggested by some further results obtained from arrestin binding studies especially with respect to nucleotide interactions. First of all, there is the observation by flash photometric studies both from light absorption and scattering changes that arrestin binds already to photoexcited rhodopsin (meta II state) prior to the phosphorylation by the rhodopsin kinase and that this binding is inhibited by ATP and not by ADP [10]. On the other hand, there is growing evidence that a permanent arrestin binding to phosphorylated meta II rhodopsin alone does not allow for its final disactivation if the amount of arrestin in the receptor cell is 8–10% of rhodopsin [21]. Therefore, if the rhodopsin bleach exceeds 10% of rhodopsin excess meta II rhodopsin should be found which cannot bind to arrestin. Thus photoactivation should partially persist which is not the case [9]. Furthermore, kinetic studies of light scattering with

ARG	ARG	GLY	ILE	ALA	LEU	ASP	GLY	LYS	ILE	LYS
(B)	(B)	(N)	(N)	(N)	(N)	(A)	(N)	(B)	(N)	(B)

Fig.5. Part of amino acid sequence of phosphoryl-binding sites from bovine arrestin (AA 290–300) and amino acid charge (N = neutral; A = acidophilic; B = basic) from Yamaki et al. [7].

stacked discs indicate that arrestin undergoes a nucleotide exchange during the binding process like transducin does [22].

The results of this study directly support the idea of an arrestin model that employs a nucleotide exchange. In the ADP form arrestin should have a high affinity to photoexcited rhodopsin but not in the ATP form. The affinity of ADP-arrestin to phosphorylated meta II rhodopsin should be higher than that of GDP-transducin. Therefore, it is concluded that the photoexcited rhodopsin R*-P is inhibited by a dynamic rather than by a static process very likely by means of a transient ATPase activity during the binding period [22]. The ATP binding shown here should reflect the ATP uptake of arrestin in its ADP form which takes place at moderate ATP concentrations already (80 mM). The ADP binding, its correlation to the ATP uptake as well as the assumed ATPase activity is subject of current studies of the arrestin-binding process.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft, Bonn, within the framework of the special research program 'Molecular Mechanism of Signal Reception'. One of us (W.G.) is indebted for a personal grant of this DFG-program. The authors like to thank Dr P. Gräber from photosynthesis research of this institute for his kind allowance to use the LKB-luminometer for ATP determinations and also for many helpful discussions about the applicability of the luciferin/luciferase system for an ATP-binding study.

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