Volume 224, number 1, 19–22 FEB 05276 November 1987

On the role of transducin GTPase in the quenching of a phosphodiesterase cascade of vision

V.Yu. Arshavsky, M.P. Antoch and P.P. Philippov

A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

Received 11 September 1987

The rate of GTP hydrolysis in the active site of transducin and that of the release of the phosphate thus formed have been measured. The former step has been found to be a rate-limiting one. The rate constant for GTP hydrolysis is equal to 0.027 s^{-1} at 23°C, and 0.07 s^{-1} at 37°C. Bedsides, it has been shown that the rate of GTPase reaction on the transducin α-subunit does not depend on the concentration of a complex of transducin β- and γ-subunits or on the presence of cGMP phosphodiesterase and a 48 kDa protein from rod outer segments. According to the results, GTP hydrolysis on transducin proceeds too slowly to account for the rapid quenching of a phosphodiesterase cascade in rod outer segments.

Photoreceptor; Transducin; GTPase; Phosphodiesterase

1. INTRODUCTION

It is known that the phosphodiesterase cascade in photoreceptor cells comprises two amplification steps [1]. At the first step the photoexcited rhodopsin activates T by catalyzing the exchange of T-bound GDP for GTP and as a result the complex of T_α and GTP dissociates from $T_{\beta\gamma}$; at the second step T_α-GTP activates PDE. In consequence, the bleaching of one rhodopsin may cause the hydrolysis of up to 10^5 cGMP molecules per s [2].

A significant property of the phosphodiesterase cascade lies in its capacity not only for rapid light-induced activation but also for a sufficiently rapid quenching after the light flash. Thus, it was shown that complete termination of cGMP hydrolysis in an ROS suspension occurs in less than 4 s after the flash [3].

It is clear that rapid quenching of the phosphodiesterase cascade should take place at both steps. At the first step, this is due to rhodopsin phosphorylation that causes a considerable decrease in the rate of T activation [4–8]. The phosphorylation effect can be apparently enhanced as a result of the binding of an ROS cytoplasmic 48 kDa protein to phosphorylated rhodopsin [9].

It is traditionally assumed that the termination of PDE activation at the second step is accounted for by the GTPase activity of T [1]. However, according to [10–12], this reaction proceeds too slowly (~ tenth of seconds) to be responsible for the rapid inactivation of the cascade. In principle, two possibilities may be postulated to overcome the above contradiction:

(i) During the hydrolysis of T-bound GTP rapid cleavage of a phosphodiester bond between the β and γ phosphates of GTP takes place and, consequently, rapid inactivation of PDE occurs, but P_i is still bound to the protein, possibly via a covalent

Correspondence address: V.Yu. Arshavsky, Moscow State University, Moscow 119899, USSR

Abbreviations: ROS, rod outer segments; PDE, cyclic nucleotide phosphodiesterase; T, transducin; T_α, T_β, T_γ and $T_{\beta\gamma}$, α, β, γ and a complex of β- and γ-subunits of transducin, respectively.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

bond [13]. At the next step, the rate limiting one, P_i is slowly released from the GTPase active site; thus the release of P_i by no means correlates with the inactivation of PDE. If this explanation is correct, it will be clear that the rate of the GTPase reaction, measured under the conditions of GTP excess over T [10,11], will only show the rate of P_i release from the active site of GTPase but will reveal nothing about the real rate of GTP hydrolysis. Moreover, even under single-turnover conditions (if T is in molar excess over GTP), which allow one to measure the rate of GTP hydrolysis in the GTPase active site, an assay of the P_i formed in the course of the reaction (as in [12]) would not provide an unequivocal answer, for there is some possibility that a relatively long-lived complex, T-P_i-GDP, where P_i is covalently bound to the protein, may be formed [13]. Therefore, we may come to an unequivocal solution only by determining the rate of GDP appearance in the active site of T under single-turnover conditions.

(ii) The known estimates for the rate of GTP hydrolysis on T apply only for in vitro conditions and do not indicate the real rate of the GTPase reaction in ROS where there may be some acceleration mechanism connected, for instance, with the effect of certain cytoplasmic components on T_α.

Here, we have investigated both options.

2. EXPERIMENTAL

ROS and photoreceptor disks were isolated in cold under dim red illumination according to [14]. The rhodopsin concentration in the preparations was determined as in [4]. Phosphorylated photoreceptor disks were obtained in conformity with the method described in [4].

T and PDE were extracted according to [15], and thereupon purified on DEAE-cellulose [16]. The final purification of both T and PDE was performed by gel filtration on a Superose 12 HP 10/30 column as in [17]. 48 kDa protein was isolated as in [18]. The protein concentration was determined as described by Bradford [19], using bovine serum albumin as standard.

The GTPase activity of T was estimated by measuring the amount of P_i and GDP formed in the reaction. In either case the measurements were performed at 23 or 37°C in 30-μl samples in 20 mM Hepes-KOH buffer (pH 7.5) containing 150 mM NaCl, 5 mM $MgCl_2$, as well as photoreceptor disks, T and GTP and the required concentration. To determine the P_i release rate, the reaction mixture was supplemented with [γ-^{32}P]GTP (0.1 μCi in a sample). The reaction was arrested by adding 10 μl of 1 mM $HClO_4$ and 0.5 ml of activated charcoal (100 mg/ml) in 50 mM K phosphate buffer (pH 7.5), cooled to 0°C. After 10 min incubation the charcoal was sedimented by centrifugation and the amount of $^{32}P_i$ in the supernatant was measured. To estimate the amount of GDP formed in the reaction, 0.1 μCi [^{14}C]GTP was added to the samples. The reaction was arrested by adding 30 μl of 0.5 M $HClO_4$, after which the mixture was neutralized with 15 μl of 1 M KOH and 15 μl of 140 mM K phosphate (pH 7.5). After 10 min incubation at 0°C, proteins and $KClO_4$ were sedimented by centrifugation and the content of [^{14}C]GDP and [^{14}C]GTP was determined in the supernatant after they had been separated on a Polyanion SI column, connected to the FPLC system (Pharmacia), as described in [20].

The rate constant of the GTPase reaction was determined under the conditions of molar excess of substrate (GTP) over enzyme (T) and, vice versa, molar excess of enzyme over substrate. In both cases the reaction was carried out in the presence of a large amount of bleached rhodopsin, as GTP binding to T proceeds in several milliseconds [21] and does not limit the rate of the GTPase reaction.

3. RESULTS AND DISCUSSION

Fig.1 shows GTPase reaction rate determination by means of the P_i increase in the reaction mixture; the measurements were made under GTP excess over T. The rate constant was found to be 0.026 ± 0.001 s^{-1} at 23°C and 0.07 ± 0.002 s^{-1} at 37°C, i.e. in good agreement with literature data [10–12]. The rate of GDP formation in the T active site was estimated under T excess over GTP (fig.2A). In this case the rate constant was equal to 0.027 ± 0.002 s^{-1} at 23°C (the same value was obtained after measurement of the P_i increase, fig.2B).

The coincidence of GTPase reaction rate constants in either case (substrate in excess over enzyme and vice versa) indicates that the total rate of this reaction is determined by GTP hydrolysis in the T active site and is not limited by P_i release

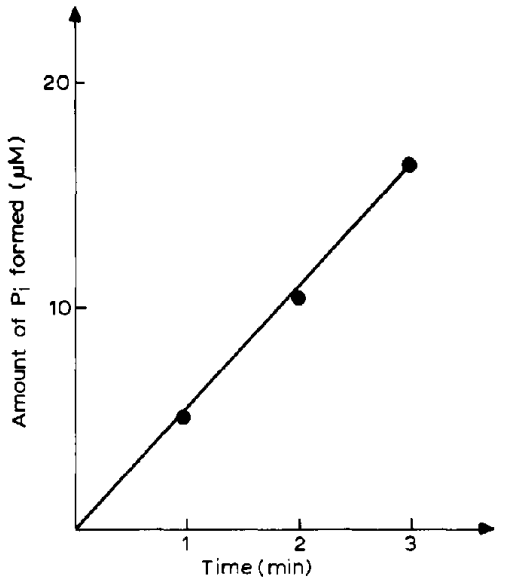

Fig.1. GTPase activity under GTP excess. The reaction mixture (see section 2) contained 3 μM T, 50 μM GTP and bleached photoreceptor disks (10 μM rhodopsin); upon incubation, the amount of formed P_i was assayed.

from the protein. Thus, in reconstituted systems containing T and photoreceptor membranes GTP hydrolysis on T does proceed too slowly to explain the rapid quenching of a phosphodiesterase cascade [3].

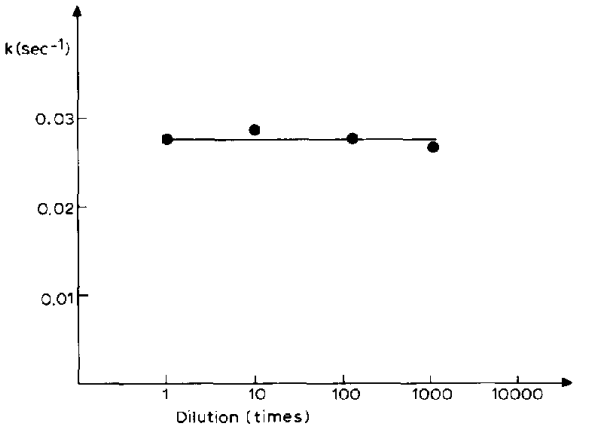

Fig.3. Dependence of the GTPase reaction rate constant on dilution of the reaction mixture. The samples (see section 2) contained 10 μM T, 8 μM GTP and completely bleached photoreceptor disks (10 μM rhodopsin); upon mixing and subsequent rapid dilution of the samples (1–2 s), the amount of formed P_i was assayed.

It is possible, however, that a photoreceptor cell has some unknown mechanism of GTPase acceleration. In conformity with this hypothesis, we verified the possibility of GTPase activation during the interaction of T_α-GTP with some ROS proteins.

From a variety of ROS proteins we selected $T_{\beta\gamma}$ complex, as well as PDE and the 48 kDa protein.

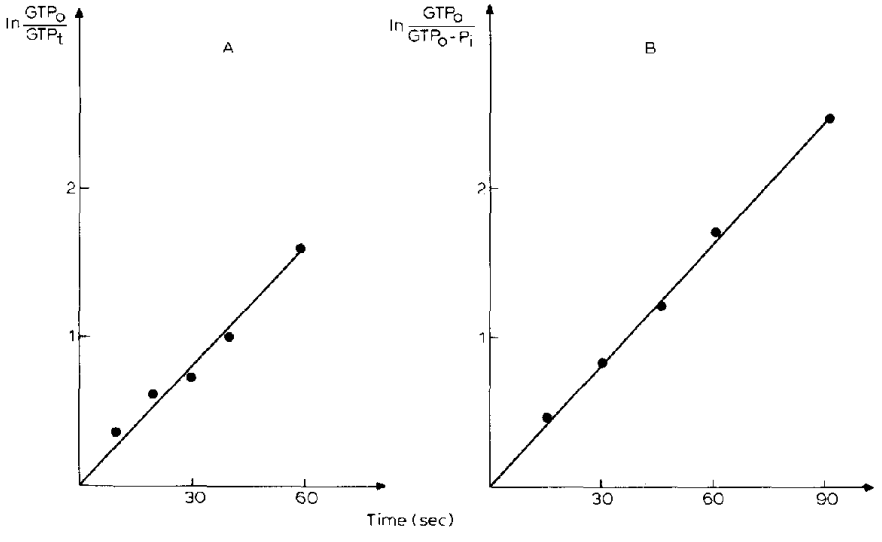

Fig.2. The determination of rate constants for the GTPase reaction under T excess, according to GDP (A) and P_i (B) formation. The reaction mixture (see section 2) contained 10 μM T, 8 μM GTP and bleached photoreceptor disks (10 μM rhodopsin).

The point is that all these proteins interact in some way with T_α or with T as a whole: $T_{\beta\gamma}$ and T_α produce a reversibly dissociating complex [11], PDE is activated by T_α-GTP [1] and the 48 kDa protein apparently competes with T for rhodopsin [9] or for PDE [22].

The effect of $T_{\beta\gamma}$ on the rate of GTP hydrolysis on T_α was studied by means of a rapid (1–2 s) dilution of the reaction mixture after the 'nucleotide charging' of T. If the reaction of GTP hydrolysis on T_α is of first order, then the dilution should not affect the rate constant. But if the GTPase activity of T_α (after it has bound GTP and dissociated from the complex with $T_{\beta\gamma}$) is sensitive to the presence of $T_{\beta\gamma}$, then a change in the measured rate constant for the GTPase reaction will be observed. As seen from fig.3, a significant dilution (up to 1200-fold) in no way affects the measured rate constant of the GTPase reaction, and consequently, $T_{\beta\gamma}$ exerts no effect on the GTPase activity of T_α.

A negative result was also obtained in studying the PDE effect on GTPase, with PDE being added to the reaction mixture in amounts equimolar to T (in the presence and absence of 1 mM cGMP). We also did not succeed in detecting any effect of the 48 kDa protein irrespective of the presence of ATP and phosphorylated rhodopsin in the system.

Thus, it it still an open question as to whether the traditional view of GTPase as a mechanism accounting for a rapid quenching of the phosphodiesterase cascade is correct. Preliminary measurement of the GTPase reaction rate in ROS preparations which were sealed during their isolation has shown that the rate constant is equal to the value obtained for the reconstituted system. So it is possible that the fast cascade quenching is caused not by the hydrolysis of T-bound GTP but by other mechanisms, e.g. by a direct inhibitory effect of the 48 kDa protein on PDE.

ACKNOWLEDGEMENTS

This study was carried out within the framework of the 'Rhodopsin' research program sponsored by Professor Yu.A. Ovchinnikov under the auspices of the USSR Academy of Sciences and Moscow State University. We wish to express our thanks to Dr A.M. Dizhoor and Dr M. Chabre for helpful advice.

REFERENCES

[1] Stryer, L., Hurley, J.B. and Fung, B.K.-K. (1981) Trends Biochem. Sci. 6, 245–247.
[2] Yee, R. and Liebman, P.A. (1978) J. Biol. Chem. 253, 8902–8909.
[3] Sitaramayya, A. and Liebman, P.A. (1983) J. Biol. Chem. 258, 12106–12109.
[4] Arshavsky, V.Yu., Dizhoor, A.M., Shestakova, I.K. and Philippov, P.P. (1985) FEBS Lett. 181, 264–266.
[5] Arshavsky, V.Yu., Dizhoor, A.M., Kaulen, A.D., Shestakova, I.K. and Philippov, P.P. (1985) Biol. Membrany 2, 5–10 (in Russian).
[6] Arshavsky, V.Yu., Antoch, M.P. and Philippov, P.P. (1986) Biol. Membrany 3, 1197–1203 (in Russian).
[7] Miller, J.L., Fox, D.A. and Litman, B.J. (1986) Biochemistry 25, 4983–4988.
[8] Sitaramayya, A. (1986) Biochemistry 25, 5460–5468.
[9] Wilden, U., Hall, S.W. and Kühn, H. (1986) Proc. Natl. Acad. Sci. USA 83, 1174–1178.
[10] Baehr, W., Morita, E.A., Swanson, R.J. and Applebury, M.L. (1982) J. Biol. Chem. 257, 6452–6460.
[11] Bennett, N. and Dupont, Y. (1985) J. Biol. Chem. 260, 4156–4168.
[12] Yamanaka, G., Eckstein, F. and Stryer, L. (1985) Biochemistry 24, 8094–8101.
[13] Chabre, M., Bigay, J., Detterre, P. and Pfister, C. (1986) in: Desensitization and Adaptation Mechanisms to Signal Molecules (Konish, T.I. ed.) Springer, Berlin.
[14] Smith, H.G., Stubbs, G.W. and Litman, B.J. (1975) Exp. Eye Res. 20, 211–217.
[15] Kühn, H. (1978) Biochemistry 17, 4389–4395.
[16] Kohnken, R.E., Eadie, D.M., Revzin, A. and McConnell, D.G. (1981) J. Biol. Chem. 256, 12502–12509.
[17] Dizhoor, A.M., Rakhilin, S.V. and Philippov, P.P. (1987) Biol. Membrany 4, 818–824 (in Russian).
[18] Wilden, U., Wüst, E., Weyand, I. and Kühn, H. (1986) FEBS Lett. 207, 292–295.
[19] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[20] Dizhoor, A.M., Arshavsky, V.Yu., Kaulen, A.D. and Philippov, P.P. (1986) Biol. Membrany 3, 691–696 (in Russian).
[21] Liebman, P.A. and Pugh, E.N. jr (1981) Curr. Top. Membranes Transp. 15, 157–170.
[22] Zuckerman, R. and Cheasty, J.B. (1986) FEBS Lett. 207, 35–41.