Association between a photo-intermediate of an M-lacking mutant D75N of *pharaonis* phoborhodopsin and its cognate transducer

by

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Abbreviations: DM, n-dodecyl-β-D-maltoside; IPTG, isopropyl-1-thio-β-galactoside; K_D, dissociation constant; n, molar ratio of ppR/t-Htr complex; *p*HtrII, *pharaonis* halobacterial transducer of *p*pR; *p*pR, *pharaonis* phoborhodopsin (*pharaonis* sensory rhodopsin II); *p*pRHis, C-terminal-His tagged *p*pR; t-Htr, truncated *p*HtrII; t-HtrHis, C-terminal-His tagged t-Htr; D75N mutant, mutant in which Asp75 is substituted by Asn; Z_{570}, red-shifted O-like intermediate of D75N (λ_{max} of 570 nm); λ_{max}, maximum wavelength.

ABSTRACT

pharaonis phoborhodopsin (or *pharaonis* sensory rhodopsin II) is a receptor of the negative phototaxis of *Natronobacterium pharaonis* and forms a complex with its transducer *p*HtrII in membranes. Flash-photolyis of a D75N mutant did not yield the M-intermediate, but an O-like intermediate is observed in a ms time range. We examined the interaction between the D75N of *p*pR and t-Htr (truncated *p*HtrII). These formed a complex in the presence of 0.1% n-dodecyl-β-maltoside, and the association accelerated the decay of the O of D75N from 15 to 56 s^{-1}. From the decay time constants under varying ratios of D75N and t-Htr, n, the molar ratio of D75N/t-Htr in the complex, and K_D, the dissociation constant, were estimated. The value of n was unity and K_D was estimated to 146 nM. This K_D value can be considered to be the association between the photo-intermediate and t-Htr, which is deduced by the method of estimation. Previously we (Photochem. Photobiol. 74. 489-494 (2001)) reported a K_D of 15 μM for the interaction between the wild-type and t-Htr by means of the change in M-decay rates. Therefore, this value should be the K_D value for the interaction between M of the wild-type and t-Htr.

INTRODUCTION

Halobacteria have four retinal proteins that are bacteriorhodopsin (bR) [1,2], halorhodopsin (hR) [3,4], sensory rhodopsin (sR, also called sensory rhodopsin I, sRI) [5,6] and phoborhodopsin (pR, also called sensory rhodopsin II, sRII) [7,8]. Although they have a high similarity with each other, their function is differentiated: bR and hR are light-driven ion-pumps while sR and pR are photoreceptors. The ground state of sR (or sRI, λ_{max} of 587 nm) is a receptor mediating positive phototaxis, whereas its long-lived photo-intermediate (S373, λ_{max} of 373 nm) acts as a receptor of negative phototaxis [5]. *pharaonis* phoborhodopsin (*p*pR or *pharaonis* sensory rhodopsin II, *p*sRII) [9-12] is a pigment corresponding to pR (or sRII) of *Natronobacterium pharaonis*. pR and *p*pR absorb maximally 487 and 498 nm light, respectively, and work as receptors of the negative phototaxis. Each receptor transmits its signals through an integral membrane halobacterial transducer protein (Htr) that is considered to form a complex firmly with the respective receptors: they are HtrI for sRI, HtrII for pR (sRII) and *p*HtrII for *p*pR (*p*sRII) [13,14]. Htr proteins have both a signaling domain and two methylatable domains [8,15] such as an aspartate receptor (Tar) in *Escherichia coli* [16], and activate a phosphotransfer

cascade producing flagella motor responses. By these signaling systems, bacterial cells move toward a longer wavelength light (λ>520 nm) where bR and hR work, while they avoid a shorter wavelength light (λ<520 nm) that contains harmful near-UV light.

Wegener et al. [17] and we [18] succeeded in an expression of a truncated *p*HtrII (named t-Htr) in *E. coli*, where t-Htr is an N-terminal sequence of 159 amino acid residues of *p*HtrII. This t-Htr has the ability to c ontact *p*pR [17-19] under solubilized conditions with n-dodecyl-β-D-maltoside (DM). It is shown that HtrI lacking the portion of methylation and signaling domains (a truncated HtrI) can interact with sRI (pR) [20]. Recently, a chimera protein composing a transmembrane part of *p*HtrII and the cytoplasmic domains of eubacteria was expressed in *E. coli*, and this chimera protein was able to receive the light signal from *p*sRII (*p*pR), which evoked the phototaxis of *E. coli* cells [21]. Htr transducers are proposed to interact phys ically and functionally with their cognate sensor pigments via helix-helix contacts between their transmembrane segments [21]. Thus, *p*pR/t-Htr (complex between *p*pR and t-Htr) in the presence of DM is considered to serve as an adequate model system to elucidate the signal transfer. Furthermore, the asso

ciation of *p*pR with t-Htr changed the photo-induced accessibility of the Schiff base by azide and hydroxylamine [22], indicating the interaction of t-Htr with the receptor pigment.

Our previous paper [18] showed that the M-decay of the complex between the wild-type *p*pR and t-Htr becomes about twice as slow as that of *p*pR alone. From the M-decay rate data during the titration of t-Htr with the wild-type *p*pR, the dissociation constant, K_D, of the complex was estimated to be 15 μM. On the other hand, Wegener [19] suggested a K_D of 100 nM in the dark. What is the reason for this great difference between 15 μM and 100 nM? One possible explanation is that the complex forms firmly in the dark (100 nM), while at the M-state, the affinity of the complex becomes weak (15 μM), because this value was estimated from the M-decay data of the complex between the wild-type *p*pR and t-Htr. This assumption underlies the present work where the interaction between the D75N mutant and t-Htr is investigated using flash-photolysis, because the D75N mutant lacks the M-intermediate and has an O (or N)-like intermediate as the only main intermediate in an ms time range [23].

MATERIALS AND METHODS

Sample preparations

Expression plasmids of D75N, D75NHis and t-HtrHis were constructed as reported previously [18,24,25]. Here His means a tag of

6 x histidine bound to the C-terminal. Proteins of D75N, D75NHis and t-HtrHis were expressed in *E. coli* BL21 (DE3). Purification of these proteins was described elsewhere [26,27]. The sample medium was exchanged by ultafiltration (UK-50, Advantech, Tokyo), and samples were finally suspended in a buffer solution containing 400 mM NaCl, 10 mM Tris-HCl (pH 7.0) and 0.1% DM (n-dodecyl-β-D-maltoside).

Flash spectroscopy

The apparatus and procedure were essentially the same as described previously [28]. A photo-intermediate of D75NHis alone and a D75NHis/t-HtrHis complex was observed at 570 nm, and its time-course was analyzed by a single exponential equation to determine the kinetic constant (for details see the Results section).

Titration of free t-Htr with D75N and estimation of binding parameters

Under the condition in which the t-HtrHis concentration was kept constant at 25 μM, varying concentrations of D75NHis were added to change the molar ratio of t-HtrHis to D75NHis, and we measured the kinetic constant of the intermediate by flash spectroscopy as described above. We estimated the binding parameters (K_D and n, where n means the number of binding sites) using the same method described elsewhere [18].

RESULTS

First, we examined the possibility of the association of (untagged) ground state D75N with t-HtrHis in the presence of 0.1% DM using Ni-NTA resins. The upper two panels of Fig. 1 (a and c) show spectra of *p*pR that were not adsorbed on the resin, while the lower (b and d) whows spectra of the adsorbed *p*pR fraction. The left two panels show *p*pR spectra ((a), unabsorbed; (b) absorbed) when only D75N was applied to the column, while the right two show *p*pR spectra ((c), unabsorbed; (d), absorbed) when t-HrHis was first adsorbed on the resin, followed by the application of D75N. Comparison between (b) and (d) clearly reveals that t-HtrHis makes the (untagged) D75N bind to the resin; D75N then interacts with t-HtrHis that binds to the resin because of the tag. This finding demonstrates the association of (ground state) D75N with the transducer even in the presence of a detergent, DM.

D75N lacks the M-intermediate during the photocycle becaus e Asp-75, the proton acceptor from the protonated Schiff base (PSB), is replaced by the neutral Asn [23]. In an ms time range, a red-shifted O (or N)-like intermediate (λmax of 570 nm) is observed. Adopting a schema by Schmies et al. [23], the photocycle of D75N may be described as: D75N*p*pR_{520} → K_{565} → Z_{570} → D75N*p*pR_{520}. The suffix indicates λ_{max}. They considered Z_{570} as a

mixture of two intermediates. The characterization of Z_{570} awaits further investigation, but it should be stressed that M does not appear and that only an O-like intermediate is observable except for the early time range after the flash. As shown in the insets in Fig. 2, the logarithmic plot suggests the presence of an earlier intermediate with a lifetime of several ms, which might be K_{565} because Schmies et al. [23] reported the half-time of 7 ms.

The decay rates of Z_{570} of D75NHis or the D75NHis/t-HtrHis complex were measured at 570 nm. Curve (a) in Fig. 2 is the decay trace of free D75NHis and curve (b), that of the D75NHis/t-HtrHis complex. Insets delineate the log of absorbance changes versus time after the flash. These traces were analyzed by a single exponential equation to estimate the time constant of Z_{570}, because the contribution from the earlier component is very small. In addition, Z_{570} is a slow component and semi-logarithmic analysis may give an exact time constant for the slower component. These clearly show the difference in the decay rate of Z_{570} between them; the rate constant of D75NHis alone was 15 ± 0.1 s^{-1} (a 47 ms half-time) while that of the complex, 56 ± 0.3 s^{-1} (a 12 ms half-time). Schmies et al. [23] described that the decay time constant of Z_{570} (without t-Htr) was 28 ms which is different from the present value. The reason is not

clear, but differences in the experimental conditions are probable: their sample was reconstituted into purple lipid membranes suspended in 20 mM NaCl while the present sample was DM-solubilized in 400 mM NaCl. The decay of Z_{570} of the complex is almost 4-fold faster than that of D75NHis alone. No change in the decay rate of Z_{570} was observed when additional t-HtrHis was added to the D75NHis/t-HtrHis complex, implying that there was no free D75NHis in the sample.

We titrated 25 μM of t-HtrHis with D75NHis and measured the decay rate of Z_{570}. The decay curve was composed of two components except for the later titration data. This is very natural because this sample may contain free D75NHis, the D75NHis/t-HtrHis complex and free HtrHis, the former two of which are active in the flash spectroscopy with different kinetic constants. Eight kinetic traces were obtained under the condition of varying molar ratios of t-HtrHis to D75NHis. All data were fitted well with an equation of $\alpha\exp(-15t) + \beta\exp(-56t)$.

We must consider the extinction coefficients of Z_{570} derived from both D75His and D75His/t-HtrHis, which were assumed to be equal. Under this assumption, α and β in the above equation are proportional to the concentration of D75NHis alone and the D75NHis/t-HtrHis complex, respectively. This assumption was deduced from the following. 1) The extinction

coefficients of the ground state of D75NHis and D75NHis/t-HtrHis are the same (data not shown). 2) In the flash-induced light-dark difference spectrum, the de flection ratios of the positive band caused by the formation of Z_{570} to the negative band caused by the depletion of the original pigments are equal (data not shown).

In Fig. 3, the free D75NHis concentration, [D75NHis], is plotted against the D75NHis/t-HtrHis concentration, [D75NHis/t-HtrHis]. From this curve, the K_D and n values were estimated to be 146 nM ($\pm$8) for K_D and 1 ($\pm$0.001) for n. When [D75NHis] was varied at 5, 10, 15, 20 and 30 μM, the same values of K_D and n were obtained. Data were shown in Fig. 4.

Discussion

This paper shows the following; 1) The ground state of D75N can interact with t-Htr even in the presence of a detergent. 2) The value of n is unity, indicating the (1:1) stoichiometry of D75N and t-Htr to be the same as that of the wild-type [18]. Zhang and Spudich [29] have demonstrated that HtrI exists as a dimer. Therefore, the stoichiometry is probably (2:2) for D75N/t-Htr as well as the wild-type/t-Htr. 3) The K_D value is 146 nM. Previously, we [18] showed a K_D of 15 μM, which was estimated from the decay rate constants of the M-intermedi ate of *p*pR and the *p*pR/t-Htr complex. This value, hence, should

be considered as the K_D at the M-state of the complex. On the other hand, Wegener [19] presented an interesting result on the interaction of the wild-type *p*pR and t-Htr. Using iterative titration calorimetry, they observed a K_D of about 100 nM in the dark. It is interesting that this value is very close to 146 nM for the intermediate of D75N that lacks M in the photocycle and is almost 100-fold smaller than that for the interaction between M of the wild-type and t-Htr.

What is the signaling state of *p*pR? Data accumulated in pR (sRII) suggest both M and O [30,31], and Spudich et al. [31] proposed that breaking the salt-bridge between C and G helices via the Schiff base is the trigger of the taxis. This proposal is supported by their observation that cells expressing the D73N mutant of sRII (corresponding to D75N of *p*pR) shows 'avoidance' behavior even in the dark [31]. Illumination of the cells showed a further avoidance reaction, suggesting that the Z_{570}-intermediate might be one of a signaling state. If one considers that this Z_{570} may correspond to the O- (or N-) of the wild-type although further characterization is required, this observation implies that O (or N) is also a signaling state. As described above, 15 μM of K_D reported previously [18] should be that for the complex between M and the transducer. Therefore, we wanted to investigate the K_D value for an interaction between O and the transducer. When we used the wild-type *p*pR, the change in the photocycling rate

constants induced by the association with t-Htr was not large, and the exact determination of the O-decay rate constants was not possible. Therefore, in this paper, we undertook investigating the interaction of the transducer with the (O-like) intermediate of D75N.

The interaction was very strong and was about 100-fold stronger than that of the M-intermediate. Wegener et al. [32] showed a photo-induced clock-wise rotation of TM2 of t-Htr, which results in a decrease in the association affinity (decoupling). This is consistent with the low affinity of the M-intermediate as was reported pr eviously [18]. The photo-induced changes in the EPR signal continued till the full recovery of the original pigment [17,32], which seems to be contradictory to the present data in which the association affinity of the O-intermediate (146 nM) is reset to the stronger value of the ground-state *pp*R (100 nM from Wegener et al.[19]), provided that Z_{570} corresponds to the O-intermediate of the wild-type. Further studies are necessary on the association affinity of each M or O (or N)-intermediate with the transducer and on the question of what is the signaling state in *ppR*, although the signaling state of pR (sRII) has been proven [30].

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FIGURE LEGENDS

Fig. 1

The D75N mutant can bind to t-HtrHis even in the presence of 0.1% DM. Purified (tag-less) D75N was applied to a Ni-NTA column. After the application, buffer W (see ref.[27]) was allowed to flow and the eluted material was collected followed by measurement of the spectrum which is shown in panel (a). After the application of D75N and extensive washing with buffer W to remove non-specifically bound proteins, the bound proteins were eluted with buffer E (see ref.[27]), and the eluted material was collected followed by measuring the spectrum which is shown in panel (b). The same experiments were done using the complex between D75N and t-HtrHis. Panel (c) shows the spectrum of the non-adsorbed fraction while (d) shows that of the adsorbed fraction.

Fig. 2

The decay rate of a photo-intermediate is different between free D75NHis (a) and the D75NHis/t-HtrHis complex (b). The decay was monitored at 570 nm. The medium contained 400 mM NaCl, 0.1% DM adjusted at pH 7.0 with 10 mM Tris-HCl. Protein concentrations of D75NHis were 5 μM for both (a) and (b). Insets shows the logarithmic plot of (a) and (b).

Fig. 3.

The concentration of the free D75NHis, [D75NHis], is plotted versus the concentration of D75His/t-HtrHis$_n$ complex, [D75NHis/t-HtrHis$_n$], during the titration, the details of which are referred to in [18]. These concentrations are expressed in the unit of μM. The closed circles are data points and the solid line is a fitting curve calculated by the method described previously [18]. The broken line indicates the interaction between M of the wild-type *pp*R and t-Htr which was obtained previously [18]. Data were regressed with non-linear regression software (Origin, Microcal, Northampton, MA) to evaluate K_D and n which were 146 μM and 1.0, respectively.

Fig. 4.

K_D and n values estimated at varying initial concentrations of t-Htr (unit of μM). The closed circles are K_D values (the left ordinate and its unit of nM) while the open circles are n (the right ordinate and its dimensionless unit). The method was the same as those in Fig. 3 except for the initial concentrations of t-Htr.

Fig.1

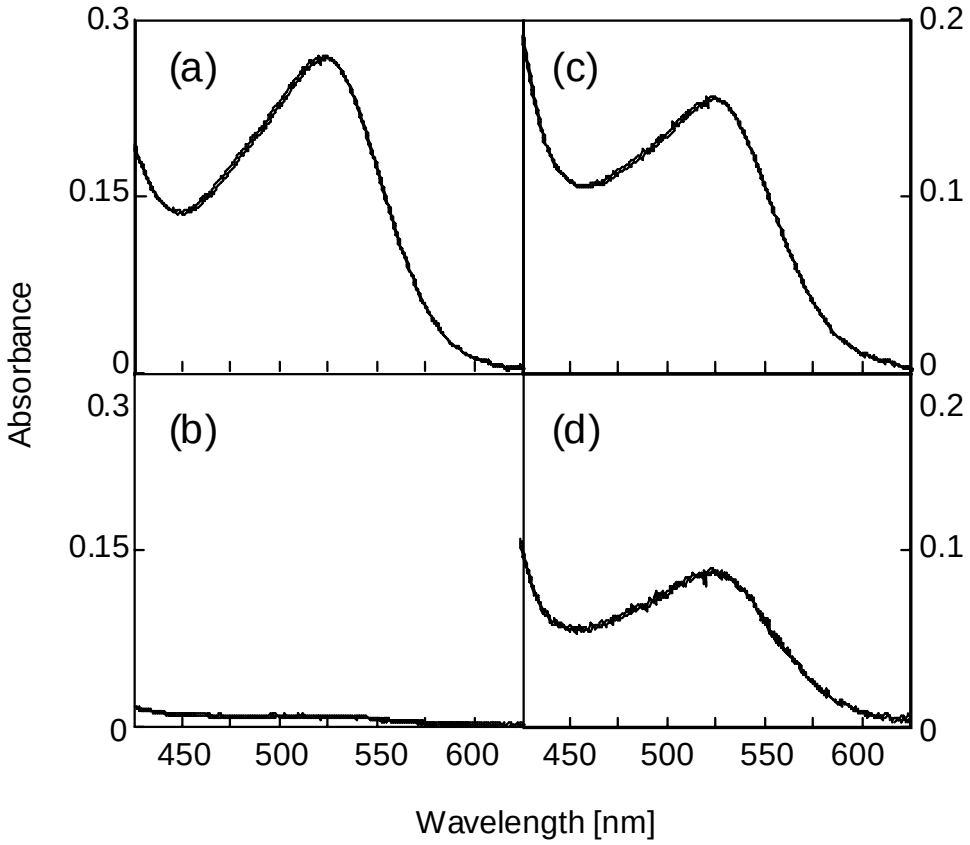

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Fig.2

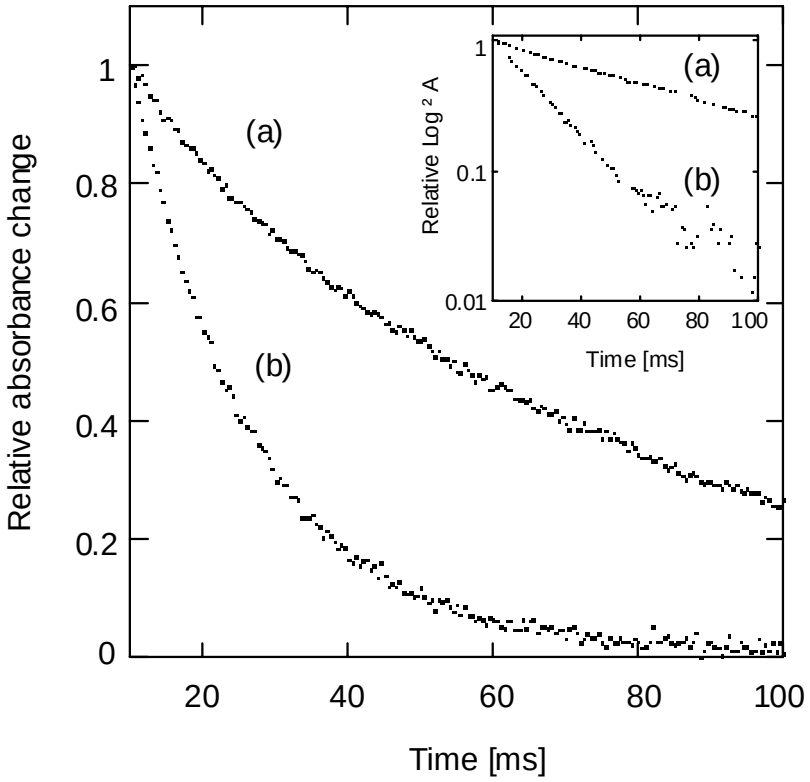

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Fig.3

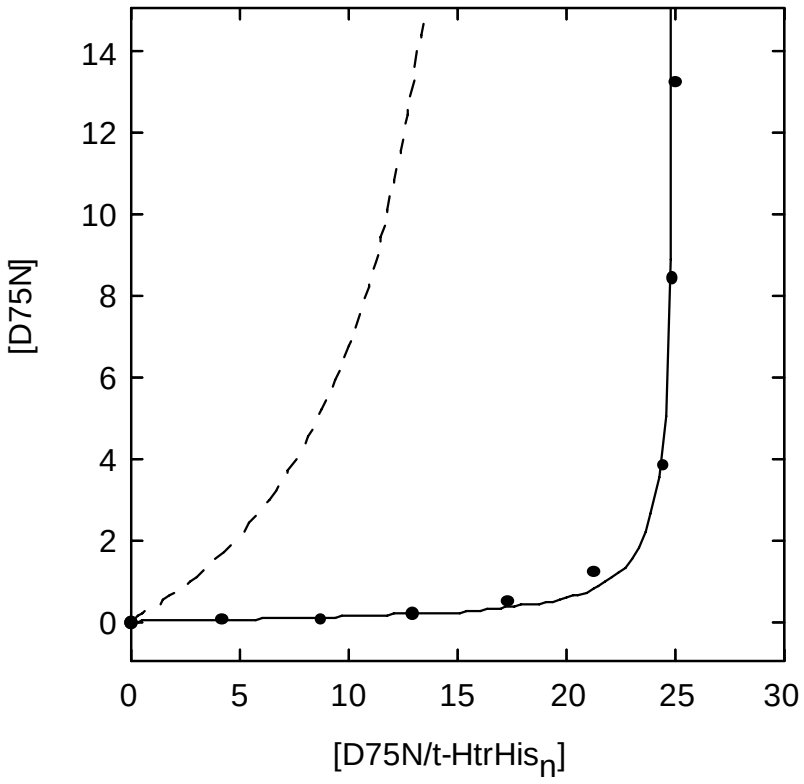

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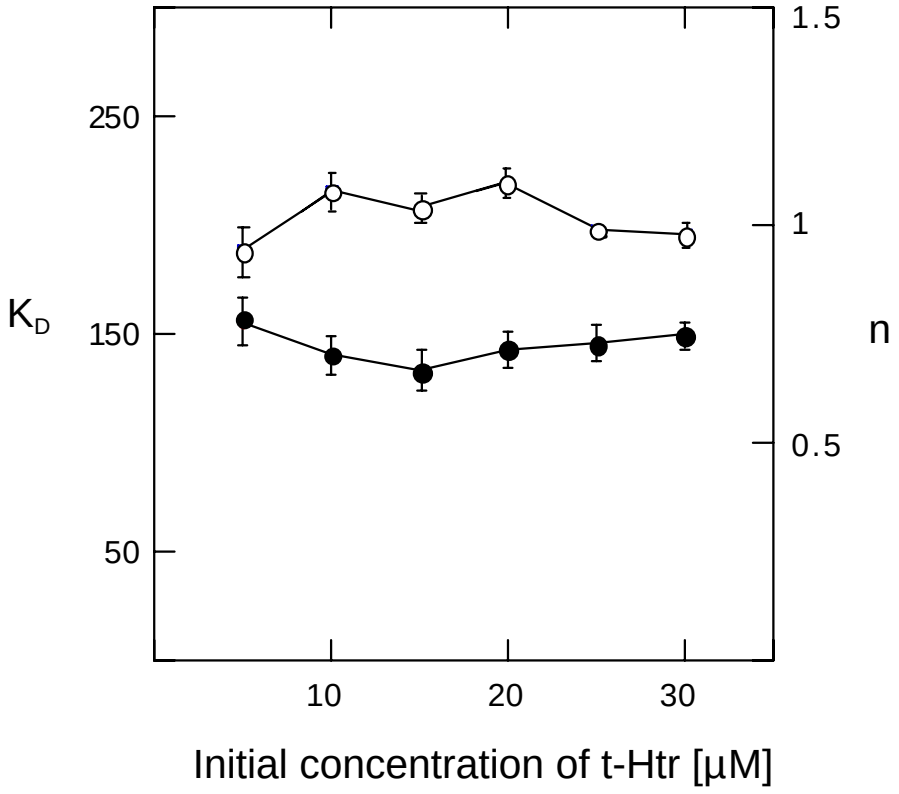
Fig.4



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