

Identification of Distinct Domains for Signaling and Receptor Interaction of the Sensory Rhodopsin I Transducer, HtrI

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The phototaxis-deficient mutant of *Halobacterium salinarum*, Pho81, lacks both sensory rhodopsin I (SR-I) and its putative transducer protein HtrI, according to immunoblotting and spectroscopic criteria. From restriction analysis and selected DNA sequencing, we have determined that the SR-I⁻ HtrI⁻ phenotype results from an insertion of a 520-bp transposable element, ISH2, into the coding region of the SR-I apoprotein gene *sopI* and deletion of 11 kbp upstream of ISH2 including the first 164 bp of *sopI* and the entire *htrI* gene. SR-I and HtrI expression as well as full phototaxis sensitivity are restored by transformation with a halobacterial plasmid carrying the *htrI-sopI* gene pair and their upstream promoter region. An internal deletion of a portion of *htrI* encoding the putative methylation and signaling domains of HtrI (253 residues) prevents the restoration of phototaxis, providing further evidence for the role of HtrI as a transducer for SR-I. Analysis of flash-induced photochemical reactions of SR-I over a range of pH shows that the partially deleted HtrI maintains SR-I interactions sites responsible for modulation of the SR-I photocycle.

Sensory rhodopsin I (SR-I) is a phototaxis receptor found in the membranes of the archaeon *Halobacterium salinarum* (22). Its gene (*sopI*) predicts a seven-transmembrane α -helix structure embedded in the membrane connected by short hydrophilic loops (1). A second protein was implicated by mutant analysis to be required for SR-I control of cell motility responses (18). On the basis of its reversible carboxymethylation and other biochemical similarities to eubacterial chemotaxis transducers, the protein was proposed to function as a halobacterial transducer for SR-I, relaying signals from the receptor to cytoplasmic components controlling the flagellar motor (21). The protein, now called HtrI, was isolated, and its gene was cloned and found to reside immediately upstream of *sopI* (27). Physical proximity of SR-I and HtrI was initially indicated by receptor chromophore migration to HtrI in experiments with radiolabeled retinal (18). Altered photoreactions of HtrI-free SR-I (8, 19) and blockage of proton transfer sites on SR-I by HtrI (14) further support a physical interaction of the two proteins.

Plasmid-directed expression of the *htrI-sopI* region in a phototaxis mutant, Pho81, phenotypically characterized as SR-I⁻ HtrI⁻, was reported to rescue SR-I-mediated phototaxis responses (27). Transformation of Pho81 by chromosomal integration of the *htrI-sopI* region and computer-assisted motility measurements confirmed the restoration of function, but delayed phototaxis responses of peak height lower than that of the wild type were noted (7). The present report characterizes the genotypic alteration responsible for the Pho81 phenotype and demonstrates that coexpression of *sopI* and *htrI* is sufficient to restore phototaxis behavior in Pho81 to the same level of response as that of the comparably grown parent strain transformed with a control plasmid. Further, deletion of the putative methylation and signaling domains of

HtrI prevents the restoration of phototaxis and localizes receptor interaction sites to the remaining N-terminal portion of the transducer.

MATERIALS AND METHODS

Restriction enzymes were purchased from Promega (Madison, Wis.), Gibco/Bethesda Research Laboratories (Gaithersburg, Md.), or New England Biolabs (Beverly, Mass.). Ligase was purchased from U.S. Biochemical Corp. (Cleveland, Ohio). Standard molecular biological techniques were used (16).

Mevinolin was a gift from A. W. Alberts (Merck, Sharp, & Dohme).

Plasmids and strains. The *Escherichia coli* expression vector pET15b (Novagen, Inc., Madison, Wis.) was expressed in the host strain, *E. coli* BL21(DE3) [F⁻ *ompT* r_B⁻ m_B⁻ (DE3)]. *E. coli* DH5 α [*supE44* Δ *lacU169* (ϕ 80*lacZ* Δ M15) *hsdR17* *recA1* *endA1* *gyrA96* *thi-1* *relA1*] was used as the host strain for cloning unless otherwise noted.

H. salinarum Pho81 (SR-I⁻ HtrI⁻ SR-II⁻) and its parent F1x15 (SR-I⁺ HtrI⁺ SR-II⁺) (24) were screened for the absence of an endogenous restriction system (17) active on pS07 (19a). The resultant strains, Pho81r⁻ and F1x15r⁻, were used for all *H. salinarum* transformations.

***htrI* expression in *E. coli* for polyclonal antibody production.** A unique *Nde*I site was engineered by PCR (15) into the initiation methionine codon of *htrI*. Primers were synthesized at the University of Texas Medical School Health Science Center, Department of Microbiology and Molecular Genetics Core Facility (Houston, Tex.). The 400-bp *htrI* sequence between primers made to sequences containing the N-terminal *Nde*I and *Bgl*II sites (CAGCATCAAGCATATGGCCGCAC AGAC and CCTCGCGGGCCGTCTCG, respectively) was amplified by PCR (MJ Research Inc., Watertown, Mass.), using 1 to 2.5 U of *Taq* polymerase (Promega). In each of 30 cycles, the template was denatured at 92°C for 1 min, and primers were annealed at 57°C for 30 s and extended for 1 min at 72°C. The 400-bp PCR-amplified product was purified from

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a 3% NuSieve (FMC Corp., Rockland, Maine) agarose gel and was digested with *NdeI* and *BglI* to yield a 374-bp fragment.

The remainder of *htrI* was prepared by digestion with *AgeI*, which recognizes a site 76 bp downstream from the *htrI* termination codon, blunting the *AgeI* 5' overhang with Klenow enzyme (Promega), and ligation to phosphorylated *BamHI* 10-mer linkers (Promega). The ligation product was digested with *BamHI* and *BglI*, producing a 1,317-bp fragment, which was ligated to the *NdeI*-*BglI* 374-bp PCR product and the *E. coli* expression vector, pET15b (digested with *NdeI* and *BamHI*), and the ligation product was used to transform competent *E. coli* BL21(DE3) (Novagen). Transformants were identified by restriction analysis, and sequences surrounding the *NdeI*, *BglI*, and *BamHI* sites were confirmed by double-stranded sequencing (Sequenase version 2.0; U.S. Biochemical).

***htrI* expression and protein isolation.** Production of HtrI was optimized, and the protein was purified by Ni²⁺-nitrilotriacetic acid resin as instructed in the pET system manual (Novagen).

E. coli-expressed HtrI was recovered in the soluble fraction after sonication in buffer containing 2 mM phenylmethylsulfonyl chloride as described previously (2) and was further purified by preparative discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12). The protein, which has a calculated molecular mass of 57,000 Da, migrated with an *M_r* of 97,000, as does HtrI expressed in *H. salinarium*. Therefore, no halobacterium-specific modification or additional component contributes to the anomalous migration, which can be attributed to the highly acidic nature of the protein as suggested previously (27). Gel slices containing the protein were sent to Cocalico Biologicals (Reamstown, Pa.) to generate polyclonal antibodies, which were partially purified by ammonium sulfate precipitation.

***htrI-sopI* expression in *H. salinarium*.** The *H. salinarium* selectable vector, pMPK54 (10), containing a *Haloflex volcanii* mevinolin resistance gene (13), an *E. coli* pBR322-derived sequence, pAT153, enabling both high-copy-number replication in *E. coli* (26) and ampicillin resistance, sequence from an autonomously replicating halobacterial plasmid, pGRB1 (6), and 70 bp of an insertion element, ISH11 (9), was used for all *H. salinarium* transformations after excision of the eubacterial 3.0-kbp pAT153 sequence with *SphI*. Removal of pAT153 prior to transformation increases the stability of the plasmid in *H. salinarium* (10). Circularization of pMPK54ΔpAT153 produced the control vector, pMPK56, which was used to transform *H. salinarium* F1x15^r. F1x15/pMPK56 was used as the phototactically wild-type control strain. Southern analysis confirmed autonomous replication and little or no recombination with the chromosome with plasmids closely related to those used in this work (11).

pSO8 was constructed by joining the following fragments: the 8-kbp *SnaBI*-*BamHI* fragment and the 1-kbp *SalI*-*SnaBI* fragment from pMPK54 and a 1.6-kbp *BclI*-*SalI* fragment isolated from an NRC-I cosmid library (N. R. Hackett, Cornell Medical School) containing the native *sopI* gene. This fragment encodes the region from amino acid residue 330 of HtrI to the C terminus of SR-I.

***H. salinarium* plasmids.** Plasmid pVJY1 (Fig. 1) contains the entire *htrI-sopI* region including 282 bp of upstream sequence and was constructed by ligating the following fragments: the 3.61-kbp *SmaI*-*HindIII* fragment from pSO8, the 6.6-kbp *BamHI*-*HindIII* fragment from pSO7 (11), and the 1.5-kbp *BamHI*-*SmaI* fragment from the cloned *htrI-sopI* region (27).

To construct pVJY5, DNA encoding residues 248 to 500 containing the putative methylation and signaling domains of

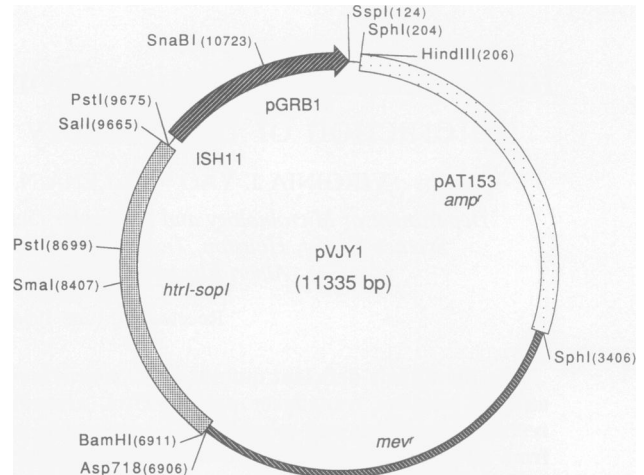


FIG. 1. Plasmid map of pVJY1 containing the *htrI-sopI* region. See Materials and Methods.

htrI cloned in pBSKSII+ was excised by digesting with *SstI*, blunting the ends with Klenow enzyme, ligating phosphorylated 8-mer *PstI* linkers (Promega), digesting with *PstI*, and ligating. The *SstI* site in the vector was previously removed by digesting with *SstI*, blunting the ends with Klenow enzyme, and ligating. The *htrI* gene was replaced in pVJY1 with *htrI* Δ*SstI*-*PstI* by digesting with *BamHI* and *AgeI* (−286 to 1687) and inserting the shortened fragment. Sequencing of the *htrI* deletion region revealed four *PstI* linkers had been added in tandem onto the blunted *SstI* site, thereby introducing the amino acid residues GCSAAALQRCSAAA between residues 247 and 501.

***H. salinarium* transformation.** *H. salinarium* was cultured and transformed as described previously (11). Two milliliters of CM/mev (medium containing 1 μg of mevinolin per ml) was inoculated with individual transformant colonies and incubated at 37°C and 240 rpm (New Brunswick) for 5 days. Seventy microliters of each 5-day culture was used to inoculate 7.0 ml of sterile CM/mev in sterile Bellco tubes for swimming behavior studies. In addition, 200 ml of sterile CM/mev was inoculated with 1 ml of the 5-day culture and grown under the same conditions to prepare membrane vesicles (18) for flash photolysis and SDS-PAGE analysis.

Motion analysis of Pho81r[−] transformants. The diluted cultures were incubated at 37°C without agitation for 2 h. Behavioral measurements were limited to 2 h after the incubation period and were done with a computerized cell tracking system (Motion Analysis, Santa Rosa, Calif.) as previously described (23), with the following modifications. Cells were imaged by infrared light on a Panasonic TR-124MA video monitor projected from a Nikon (Nikon Inc. Instrument Group, Melville, N.Y.) Diaphot TMD inverted microscope fitted with a Leitz L32/0.40P UTK 50/0.63 objective via a Cohu camera. Stray light was diminished by blacking out the Plexiglas microscope stage housing with black construction paper and black tape, and the temperature within the housing was maintained at 37°C (Nikon NP-2 incubator). Photostimuli were delivered to the cells, and data were collected at five frames per s for 2 s prestimulus and 9 s poststimulus. Light stimuli were delivered to the cells 16 times per data set with a 45-s interval between stimuli. Data were processed in real time by the Expertvision system (Motion Analysis), using a laboratory program developed by Sundberg et al. (23) with subse-

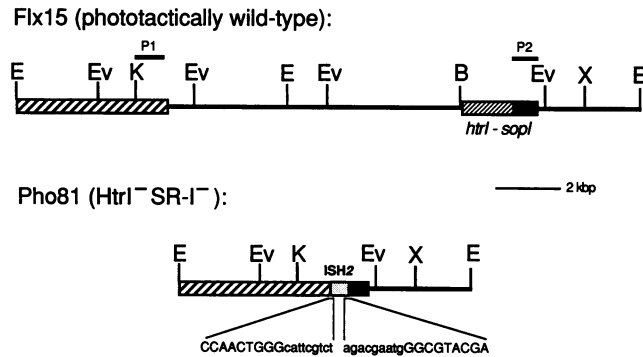


FIG. 2. Restriction maps of chromosomal DNA from Flx15 and Pho81. Probes used for Southern hybridization, P1 and P2, are, respectively, a 680-bp *KpnI-BclI* fragment and 720-bp *AatII-NotI* fragment homologous to the regions indicated. The sequences indicated on the Pho81 map show the ISH2-chromosomal DNA junctions. Bases in lowercase from left to right are the 5' and 3' ends of ISH2, respectively, and bases in uppercase represent the flanking chromosomal DNA. E, *EcoRI*; K, *KpnI*; B, *BamHI*; Ev, *EcoRV*; X, *XhoI*.

quent modifications by Takahashi (25), in a Sun SPARC-IPC workstation (Sun Microsystems, Mountain View, Calif.).

Flash photolysis of membrane vesicles. Membrane envelope vesicle preparations at ~10 mg of protein per ml were diluted 1:3 with 4 M NaCl-25 Tris-Cl (pH 6.8) in 1-cm-path-length polystyrene cuvettes (Thomas Scientific, Swedesboro, N.J.), and flash-induced absorbance changes were measured in a laboratory-constructed cross-beam kinetic flash spectrometer (20). The pH dependence of the rate of S_{373} decay was measured as described previously (19).

RESULTS

Genotypic basis of the SR-I⁻ HtrI⁻ phenotype of mutant Pho81. The basis of the SR-I⁻ HtrI⁻ phenotype of mutant strain Pho81 was investigated by restriction analysis, Southern hybridization, and selective sequencing. A 520-bp transposable element, ISH2, previously identified as inserted at the *bop* locus in *Pum⁻* mutants (4), is inserted at bp 1775 (according to the numbering of the *htrI-sopI* coding region in reference 27). The region immediately upstream of ISH2 in Pho81 is located approximately 11 kbp upstream of the *sopI* gene in Flx15, the parent strain of Pho81 (Fig. 2). The 11 kbp including the N-terminus-coding region of *sopI* and the entire *htrI* gene have apparently been deleted in Pho81, as judged by (i) comparison of the restriction map of Pho81 with that of Flx15 (Fig. 2), (ii) sequence analysis (Fig. 2), and (iii) the lack of hybridization of the *BamHI-PstI* fragment (-283 to 1505 [27]) to Pho81 genomic DNA in Southern blots (data not shown). The insertion-deletion event described here is consistent with the restriction data indicating a complex rearrangement in Pho81 observed by Ferrando-May et al. (7).

Plasmid-mediated coexpression of HtrI and SR-I. Western blot (immunoblot) analysis of membrane proteins separated by SDS-PAGE from Pho81r⁻/pVJY1Δ vesicle preparations by using anti-HtrI polyclonal antibodies demonstrated higher levels of HtrI expression than in Flx15 (Fig. 3, lanes e to g). In vivo labeling of transformants with L-[methyl-³H]methionine showed that the amount of methylation associated with HtrI expressed from the pVJY1Δ construct is also greater than that observed for the wild-type strain, Flx15, but similar to that observed for HtrI expressed in Flx5R, an overproducer of HtrI (20) (Fig. 3, lanes a to d).

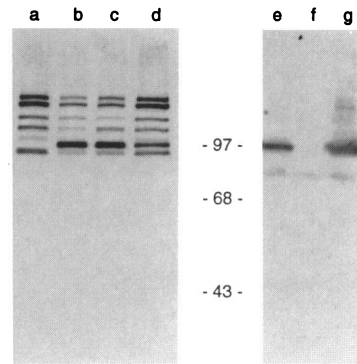


FIG. 3. Lanes a to d, Autoradiogram of methyl-³H-labeled membrane proteins separated by SDS-PAGE from strains Pho81r⁻, Flx5R, Pho81r⁻/pVJY1Δ, and Flx15r⁻, respectively. Positions of molecular mass markers are shown at the right in kilodaltons. Lanes e to g, Western blot of membrane proteins from Flx15, Pho81, and Pho81r⁻/pVJY1Δ, respectively, with an anti-HtrI antibody.

Flash photolysis of membrane vesicles prepared from Pho81r⁻/pVJY1Δ indicated that SR-I is also produced and that its flash-induced absorption difference spectrum is restored (Fig. 4) and is of greater amplitude than that observed in the wild-type Flx15 membrane vesicle preparations.

Restoration of Pho81 phototaxis responses. The expression plasmid pVJY1Δ carries native *htrI* and *sopI* preceded by their promoter region. Computerized cell tracking and motion analysis of the swimming behavior of Pho81r⁻/pVJY1Δ showed that SR-I-mediated phototaxis is restored. Reversal responses to removal of SR-I attractant light and to delivery of

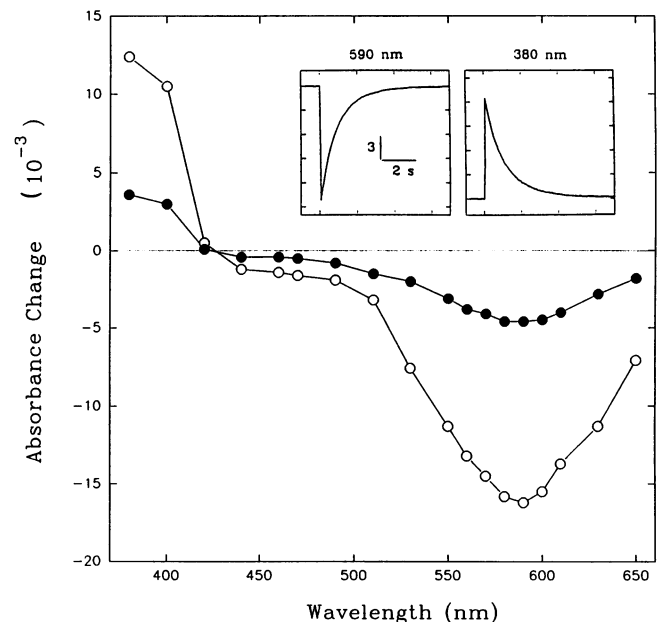


FIG. 4. Membrane envelope vesicles were subjected to a 1-ms flash of 550 ± 20 - or >620 -nm light as described previously (19), and absorption transients were monitored at various wavelengths. ○, Pho81r⁻/pVJY1Δ; ●, Flx15r⁻/pMPK56. Points are absorbance change amplitudes at 50 ms following the flash. Insets, flash-induced transients of the Pho81r⁻/pVJY1Δ preparation at the indicated wavelengths. Scale shown in the 590-nm panel applies to both panels.

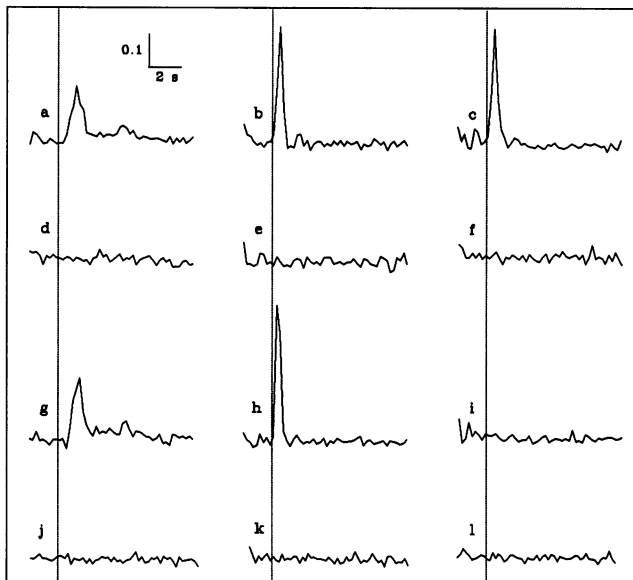


FIG. 5. Swimming reversal frequencies measured by computerized cell tracking. (a to c) *F1x15r*⁻/*pMPK56*; (d to f) *Pho81r*⁻/*pMPK56*; (g to i) *Pho81r*⁻/*pVJY1Δ*; (j to l) *Pho81r*⁻/*pVJY5Δ*. Cell populations were subjected to removal of SR-I attractant (600-nm) light for 2 s (a, d, g, and j), a 500-ms pulse of SR-I repellent (370-nm) light with 600-nm background illumination (b, e, h, and k), or a 500-ms pulse of SR-II repellent (450-nm) light (c, f, i, and l). Stimuli were initiated at the time indicated by the vertical dotted lines (2 s after data acquisition was initiated). Scale applies to all traces; vertical scale is fraction of the population exhibiting reversals per 200 ms.

SR-I repellent light by *Pho81r*⁻/*pVJY1Δ* were similar to those elicited from the wild-type strain, *F1x15r*⁻/*pMPK56*, whereas no responses were detected in *Pho81r*⁻ cells (Fig. 5). As expected from the absence of SR-II in *Pho81r*⁻, application of SR-II repellent light (Fig. 5c to l) did not elicit a behavioral response in either *Pho81r*⁻/*pVJY1Δ* or the *Pho81r*⁻ control, whereas the wild-type strain exhibited a typical response.

Effects of excision of residues 248 to 500 from HtrI. Plasmid *pVJY5Δ* carries native *sopI* and an *htrI* gene deleted of its putative methylation and signaling domain. Although the level of SR-I expression was comparable to that observed in the *Pho81r*⁻/*pVJY1Δ* transformant, *pVJY5Δ* transformants did not exhibit SR-I-mediated phototaxis (Fig. 5). This result is as predicted, since the deleted region had been proposed to contain the signaling domain (27). Flash photolysis of SR-I in membrane vesicles prepared from *Pho81r*⁻/*pVJY5Δ* cells shows a normal photochemical reaction cycle and normal yield of *S*₃₇₃, and the decay rate of the photointermediate species, *S*₃₇₃, is characteristic of SR-I complexed with HtrI (Fig. 6). *S*₃₇₃ decay is independent of pH, unlike SR-I expressed in the absence of HtrI (Fig. 6).

DISCUSSION

The plasmid-directed expression of HtrI in the *Pho81r*⁻/*pVJY1Δ* transformants was confirmed by Western blot analysis and in vivo carboxymethylation with L-[methyl-³H]methionine. Analysis of the swimming behavior of these transformants and flash photolysis of *Pho81r*⁻/*pVJY1Δ* vesicle preparations show that both the physiological response to SR-I photostimulation and the SR-I photochemical reaction cycle are fully restored. We did not observe the reduced phototaxis responses to

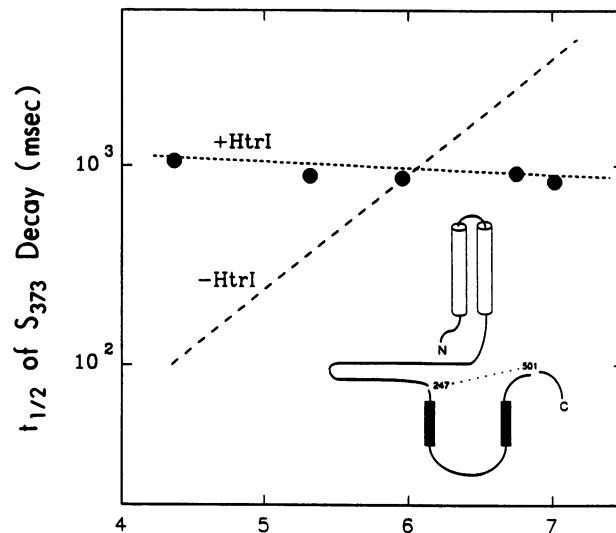


FIG. 6. Rates of thermal decay of *S*₃₇₃ in *Pho81r*⁻/*pVJY5Δ* membranes. ●, half-times established by curve-fitting as described previously (19). Regression lines are shown to the values for HtrI-free SR-I (dashed line) and HtrI-complexed SR-I (dotted line) from reference 19. The diagram illustrates the internal deletion of HtrI encoded by *pVJY5Δ*.

satürating stimuli suggested to be due to a difference in efficiency of signal amplification between a similar *htrI-sopI*-transformed *Pho81* and a phototactic control (7). We conclude expression of the *htrI-sopI* pair is sufficient to fully rescue phototaxis in *Pho81*.

The greater amplitudes observed in the depletion of *SR*₅₈₇ and the production of *S*₃₇₃ in *Pho81r*⁻/*pVJY1Δ* membrane preparations indicate that approximately five times more SR-I is expressed in the transformant than in the wild-type strain (Fig. 4). SDS-PAGE of solubilized membranes indicates that the increased level of SR-I is accompanied by a similar increase in HtrI content. No transducer-free SR-I is detected by flash photolysis, using the criteria established in reference 19. Hence, this expression system is useful for producing increased amounts of transducer-complexed SR-I.

The results of *pVJY5Δ* expression show that the region(s) of HtrI which modulates the formation and decay of light-induced conformational substates of SR-I is distinct from the methylation and signaling domains contained in residues 248 to 500. *S*₃₇₃ decay in transducer-free SR-I exhibits a pronounced pH dependence in the range from pH 4 to 8, whereas transducer-complexed SR-I is pH insensitive in this range (19). This difference provides a sensitive test for HtrI-SR-I interaction. The N-terminal half of HtrI encoded by *pVJY5Δ* is sufficient to restore normal photocycling and pH insensitivity of *S*₃₇₃ decay, and the rate is the same as for native transducer-complexed SR-I. Our interpretation is that HtrI contains at least two modular domains: one within residues 2 to 247 required for receptor interaction and a second in residues beyond residue 247 for interaction with cytoplasmic signal pathway components.

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