An Opsin Mutant with Increased Thermal Stability†

Guifu Xie, Alecia K. Gross, and Daniel D. Oprian*

Department of Biochemistry and Volen Center for Complex Systems, Brandeis University, Waltham, Massachusetts 02454

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ABSTRACT: This report describes the biochemical characterization of a double mutant of rhodopsin (N2C,D282C) in which Cys residues engineered into the protein at positions 2 (in the amino-terminal extracellular domain) and 282 (in the extracellular loop between transmembrane helices 6 and 7) are shown to form a disulfide bond and increase the thermal stability of the unliganded or opsin form of the protein. Wild-type opsin does not survive detergent solubilization and purification at pH 7.5 and 25 °C. In contrast, the N2C,D282C mutant opsin survives the purification protocol and loses less than 50% activity after incubation for 20 days under the same conditions. Less than 5% is lost after 20 days at 4 °C. While the disulfide bond clearly has a dramatic effect on protein stability, it has a minor impact on the activity of the pigment. The MII lifetime of the mutant (6.6 min) is similar to that of the wild type (7.9 min), and the specific activity of the light-activated mutant for activation of transducin is within 20% of the wild-type activity. Therefore, it seems likely that the disulfide bond does not perturb greatly the structure of the protein. For these reasons, we anticipate that the mutant may be of use in detailed kinetic and mechanistic investigations of the ligand binding reaction and for crystallization trials involving recombinant rhodopsin, especially the unliganded opsin form of the protein.

One of the surprises to come from the X-ray crystal structure of rhodopsin was the realization that the extracellular domain of the protein is very highly structured (although see ref *1*) and that the bound 11-*cis*-retinal chromophore is not visible from the outside of a space-filling model of the protein (*2*, *3*). While ligands for other GPCRs are thought to enter the receptors through their extracellular domains, the elaborate extracellular structure and hidden ligand in rhodopsin have prompted speculation that retinal does not enter the protein via the extracellular domain (*4*). In an effort to elucidate the ligand entry pathway, we have engineered a number of disulfide bonds in rhodopsin with the hope that some of these might have a large effect on binding and release of retinal from the active site (*5*). While that goal has not yet been achieved, we found that the engineered disulfide in one of these mutants (N2C,D282C)[1] dramatically increased the stability of the protein with little adverse effect on activity. We report here the biochemical characterization of this mutant with the hope that it may be of use for studies of retinal binding to opsin and crystallization of the recombinant protein.

EXPERIMENTAL PROCEDURES

Materials. 11-*cis*-Retinal (∼0.5 g) was synthesized according to published procedures (*6*) and purified first by chromatography on silica gel (70−230 mesh from Aldrich) using a 15% ether/85% hexane mixture, and then by crystallization from petroleum ether. Identification of the 11-*cis* isomer was confirmed by ^{1}H NMR as described previously for retinylamine analogues (*7*). Purity was judged to be 99.9% by HPLC (silica column developed with a 10% ether/hexane mixture and monitored by absorbance at 380 nm). The melting point was 62.2−62.8 °C. An absorption coefficient of 24 935 M^{-1} cm^{-1} at 376.5 nm was used to determine the concentration of ethanol stocks of retinal. *N*-Propyl 11-*cis*-retinylidiene (the *n*-propyl Schiff base of 11-*cis*-retinal) was prepared from 11-*cis*-retinal and *n*-propylamine as described by Zhukovsky et al. (*8*). Thermolysin, DTT,[2] sodium acetate, tris(hydroxymethyl)aminomethane (Tris), and glycine were from Sigma. Oligonucleotides for cassette mutagenesis were from QIAGEN Operon (Alameda, CA). Dodecyl β-D-maltoside (DDM) was from Calbiochem (La Jolla, CA).

The anti-rhodopsin monoclonal antibody 1D4 (*9*, *10*) was purified from hybridoma culture medium (National Cell Culture Center, Minneapolis, MN) by ammonium sulfate precipitation and ion exchange chromatography on DE-52 (Sigma, St. Louis, MO). The 1D4-Sepharose 4B immunoaffinity matrix used for purification of rhodopsin from transfected COS cells was prepared as previously described (*11*). Peptide I (DEASTTVSKTETSQVAPA), corresponding to the 18 carboxy-terminal amino acids of rhodopsin, was

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* To whom correspondence should be addressed. Phone: (781) 736-2322. Fax: (781) 736-8487. E-mail: oprian@brandeis.edu.

[1] Point mutations are indicated by the single-letter code for the wild-type amino acid, followed by the number of the amino acid, followed by the single-letter code for the new amino acid.

[2] Abbreviations: MII, metarhodopsin II; Con A, concanavalin A; DTT, dithiothreitol; βME, β-mercaptoethanol; DDM, dodecyl β-D-maltoside.

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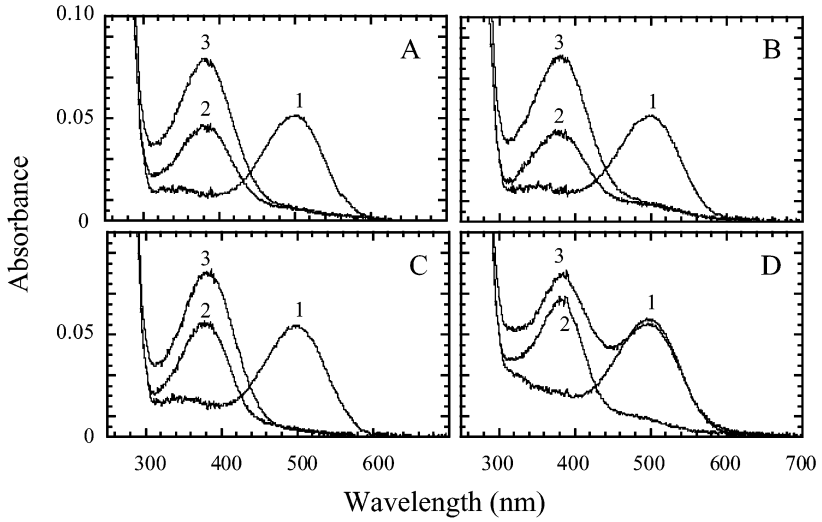

FIGURE 1: Ability of wild-type and mutant rhodopsins to be reconstituted with 11-*cis*-retinal following exposure to light and thermal decay of metarhodopsin II: (A) wild-type rhodopsin, (B) N2C single mutant, (C) D282C single mutant, and (D) N2C,D282C double mutant. In each case, spectrum 1 is the absorption spectrum of the purified pigment, spectrum 2 is the spectrum following exposure to light from a 300 W tungsten bulb filtered through a 480 nm cut-on filter for 1 min and a 1 h incubation in the dark, and spectrum 3 is the spectrum 3 min after the addition of 1−2 equiv of 11-*cis*-retinal. Spectra 3 were stable for at least 1 h (not shown). The concentrations of the purified pigments were approximately the same as judged by their absorbance at 280 nm.

purchased from American Peptide Co., Inc. (Santa Clara, CA). Peptide I contains the sequence for the 1D4 epitope and was used for elution of rhodopsin from the 1D4-Sepharose 4B matrix.

Frozen bovine retinae were from Schenk Packing Co., Inc. (Stanwood, WA). Transducin was purified from bovine retinae as described previously (*12*) and was monitored for purity by gel electrophoresis and for contamination with rhodopsin by Western blot analysis with the 1D4 antibody. [^{35}S]GTPγS (1156 Ci/mmol) was from Perkin-Elmer (Boston, MA), and nonradiolabeled GTPγS (tetralithium salt) was from Amersham Biosciences (Piscataway, NJ).

The K296G mutant opsin (*8*) and split rhodopsin mutant SR(1−5/6−7)[3] (*12*) have been described in previous publications.

Mutagenesis, Expression, and Purification of the Proteins. Procedures for mutagenesis of the synthetic opsin gene, expression in COS cells, reconstitution with 11-*cis*-retinal, and purification of the pigments have been described previously (*13*−*15*). Briefly, COS cells were transfected transiently using DEAE-dextran. Cells were harvested 72 h after transfection. Pigments were reconstituted with retinal in intact cells and then solubilized with 1% (w/v) DDM in PBS [10 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl]. The pigments were then purified by immunoaffinity chromatography on 1D4-Sepharose 4B in PBS containing 0.1% (w/v) DDM. All procedures up to and including binding of the pigment to the immunoaffinity matrix were performed at 4 °C. Subsequent washing of the column and elution with synthetic peptide were carried out at room temperature. The apoprotein forms (opsins) were purified by an identical procedure except that the cells were not incubated with 11-*cis*-retinal and all manipulations were performed under normal room lighting instead of dim red lights (Kodak #2 Safelight filters).

Digestion with Thermolysin. Digestion of rhodopsin with thermolysin was carried out essentially as described by Palczewski et al. (*16*). Briefly, rhodopsin at 4.5 μM (0.18 μg/μL) was treated with 25% thermolysin (with protease or with rhodopsin) in a final volume of 20 μL of 20 mM Tris-HCl buffer (pH 7.5) containing 2 mM $CaCl_2$ at room temperature for 15 h in the dark. A 2.4 μL aliquot containing 0.4 μg of rhodopsin was then applied to a SDS−PAGE gel for Western blot analysis.

Western Blot Analysis. The purified proteins were visualized on Western blots of 12% polyacrylamide gels (*17*) by probing for the N-terminal fragments with biotinylated Con A, similar to the method of Azen and Yu (*18*) and Clegg (*19*), as has been described previously (*20*), or by probing with the monoclonal antibody 1D4 which recognizes the nine C-terminal amino acids of rhodopsin (*9*, *10*).

Absorption Spectroscopy. UV−visible absorption spectra were recorded using a Hitachi model U-3210 spectrophotometer that was specifically modified by the manufacturer for use in a darkroom. Data were collected with an IBM-compatible microcomputer using Spectra Calc software from Galactic Industries Corp. (Salem, NH). All spectra were recorded on samples with a path length of 1.0 cm and at 25 °C. Pigments were bleached by exposure to light for 1 min from a 300 W tungsten bulb filtered through a 480 nm cut-on filter.

Transducin Activation Assays. The ability of receptors to catalyze the exchange of radiolabeled [^{35}S]GTPγS for bound GDP in transducin was monitored using a filter binding assay as has been described previously (*8*).

Time Course of Metarhodopsin II Decay. The rate of metarhodopsin II decay was measured by taking advantage of the fact that 11-*cis*-retinal binds to opsin much faster than

[3] SR(1−5/6−7) refers to an opsin that has been assembled by coexpression of two plasmids encoding fragments SR(1−5) and SR-(6−7). SR(1−5) refers to an N-terminal fragment of the protein containing the first five transmembrane segments (Met1−Ser240). SR-(6−7) refers to the C-terminal fragment containing the last two transmembrane segments (an initiator Met followed by Ala241−Ala348).

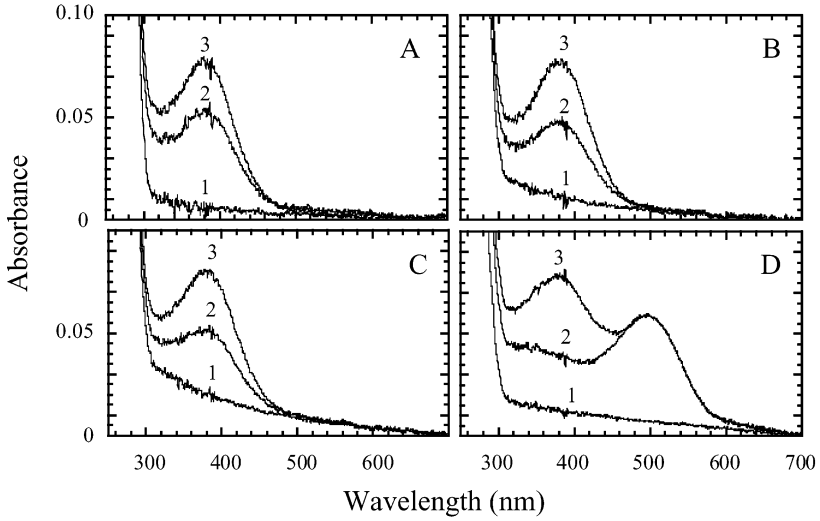

FIGURE 2: Ability of purified wild-type and mutant opsins to bind 11-*cis*-retinal: (A) wild-type opsin, (B) N2C single mutant, (C) D282C single mutant, and (D) N2C,D282C double mutant. In each case, spectrum 1 is the spectrum of the purified opsin form of the protein, spectrum 2 is the absorption spectrum 3 min after addition of ~1 equiv of 11-*cis*-retinal, and spectrum 3 is the spectrum 3 min after addition of an additional ~1 equiv aliquot of 11-*cis*-retinal. The concentrations of the purified pigments were approximately the same as judged by their absorbance at 280 nm.

metarhodopsin II decays (*21*−*23*). Thus, we measured the rate of metarhodopsin II decay as follows. The purified pigment was first isolated in 2 mM sodium phosphate buffer (pH 6.4) containing 7.5 mM NaCl and 0.1% (w/v) DDM (*24*). An absorption spectrum was recorded for the dark state of the pigment. The sample was then exposed to light from a 300 W tungsten bulb filtered through a 480 nm cut-on filter for 1 min and the spectrum of the resulting metarhodopsin II (*25*) recorded. 11-*cis*-Retinal (2−3 equiv) was added, and spectra were recorded continuously until no further absorbance increase was observed at 500 nm. Since 11-*cis*-retinal binds to opsin much more rapidly than all-*trans*-retinal is released from metarhodopsin II, opsin does not accumulate in the experiment. As a consequence, there is very little denaturation of the protein and the rate of pigment regeneration corresponds to the rate of decay of metarhodopsin II. Rate constants for the reaction were determined from the semilogarithmic plots presented in the insets of Figure 8. ΔA in the figure corresponds to the normalized absorbance change monitored at 500 nm with time; $\Delta A = (A_\infty - A)/(A_\infty - A_0)$, where A is the absorbance recorded at 500 nm for each time point, A_∞ is the absorbance at time $t = \infty$, and A_0 is the absorbance at time $t = 0$.

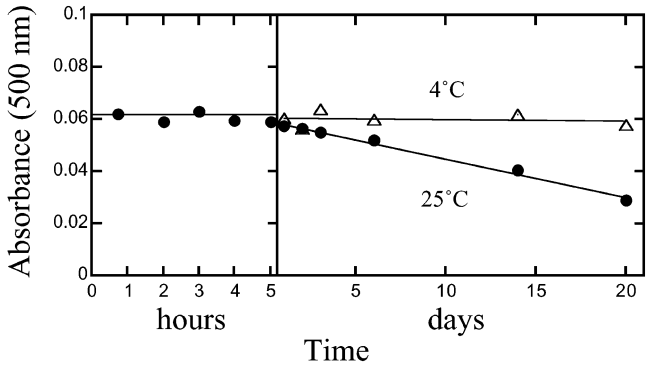

FIGURE 3: Stability of the N2C,D282C double mutant opsin. The N2C,D282C double mutant opsin was purified by immunoaffinity chromatography on 1D4-Sepharose 4B as described in Experimental Procedures. The protein was eluted from the matrix with Peptide I at time $t = 0$ and then incubated in PBS containing 0.1% (w/v) DDM and 2 mM NaN_3 for various times at 25 °C (●) as indicated in the figure before being tested for the ability to bind 11-*cis*-retinal and form a pigment. At each time point, ~3 equiv of 11-*cis*-retinal was added to the purified opsin and an absorption spectrum recorded to determine the yield of pigment. Under identical conditions, the wild-type opsin yielded no pigment even at the earliest time points. The stability of the mutant opsin at 4 °C was also monitored (△).

RESULTS

When rhodopsin is bleached by exposure to light and then incubated at room temperature and pH 7.5 for 1 h, the pigment cannot be regenerated by addition of 11-*cis*-retinal (Figure 1A) because of the rapid and irreversible denaturation of the apoprotein opsin (formed upon hydrolysis and release of all-*trans*-retinal) under these conditions. In striking contrast, the N2C,D282C double mutant fully regenerates pigment with 11-*cis*-retinal when similarly treated (Figure 1D), indicating that the mutant opsin is not denatured under these conditions (wild type and the N2C,D282C double mutant decay to opsin and all-*trans*-retinal with similar kinetics after bleaching; see Figure 8). Neither of the single mutants, N2C and D282C, could form a pigment with 11-*cis*-retinal after the 1 h incubation (panels B and C of Figure 1, respectively), showing that the double mutant is required and suggesting that a disulfide bond between the two Cys residues is responsible for the increased stability.

Given the increased stability, we wondered if the double mutant opsin might survive the procedure we use for purification of rhodopsin from transfected COS cells. As is shown in Figure 2A, wild-type opsin does not survive the purification procedure and cannot form a pigment with added 11-*cis*-retinal. The N2C and D282C single mutant opsins also do not survive the purification procedure (panels B and C of Figure 2, respectively). In contrast, the N2C,D282C double mutant opsin does survive the purification procedure and can form a pigment with 11-*cis*-retinal (Figure 2D). Importantly,

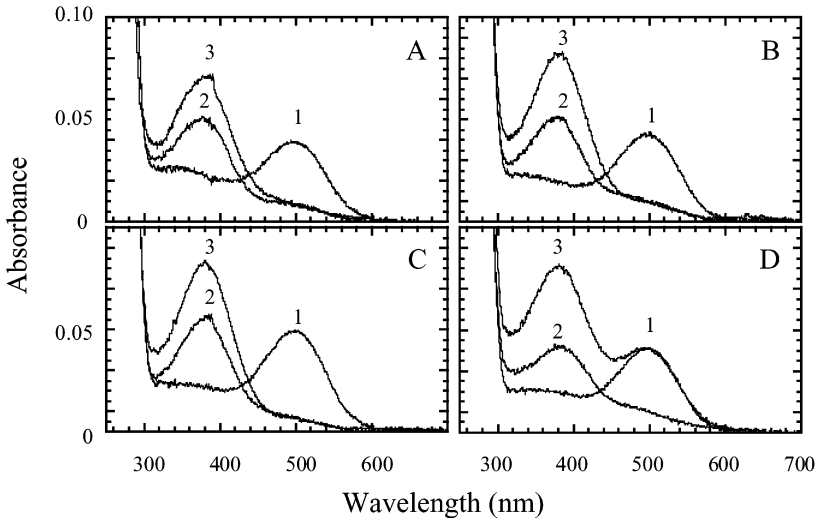

FIGURE 4: Ability of SR(1−5/6−7) wild-type and mutant rhodopsins to be reconstituted with 11-*cis*-retinal following exposure to light and thermal decay of metarhodopsin II: (A) SR(1−5/6−7) wild-type rhodopsin, (B) SR(1−5/6−7) N2C single mutant, (C) SR(1−5/6−7) D282C single mutant, and (D) SR(1−5/6−7) N2C,D282C double mutant. In each case, spectrum 1 is the absorption spectrum of the purified pigment, spectrum 2 is the spectrum following exposure to light from a 300 W tungsten bulb filtered through a 480 nm cut-on filter for 1 min and a 1 h incubation in the dark, and spectrum 3 is the spectrum 3 min after the addition of 1−2 equiv of 11-*cis*-retinal. Spectra 3 were stable for at least 1 h (not shown).

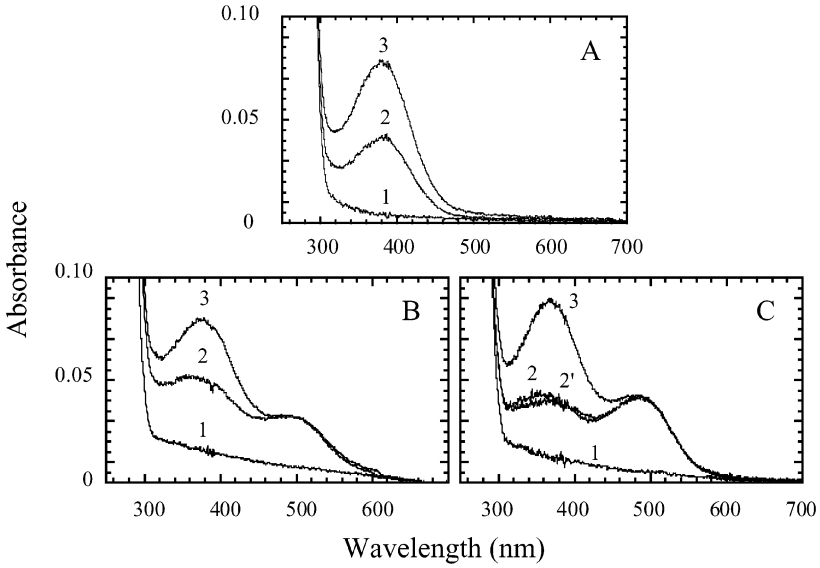

FIGURE 5: Ability of mutant opsins to bind 11-*cis*-retinal: (A) SR(1−5/6−7) opsin, (B) SR(1−5/6−7) N2C,D282C double mutant, and (C) N2C,D282C,K296G triple mutant. In each case, spectrum 1 is the spectrum of the purified opsin form of the protein, spectrum 2 is the absorption spectrum 3 min after addition of ∼1.5 equiv of 11-*cis*-retinal (or in panel C, *n*-propyl 11-*cis*-retinylidene), and spectrum 3 is the spectrum 3 min after addition of an additional ∼1.5 equiv aliquot of 11-*cis*-retinal. In panel C, spectrum 2′ was recorded 20 min after addition of the first aliquot of *n*-propyl 11-*cis*-retinylidene. The small shift in the UV absorption maximum of the sample from ∼360 to 380 nm is a consequence of hydrolysis of the excess Schiff base not bound to the protein.

the amount of pigment regenerated after purification of the N2C,D282C opsin is comparable to that obtained by purification of the pigment directly (not shown), indicating that none of the opsin has denatured during the 4 h required for the procedure. In fact, as can be seen in Figure 3, less than 50% of the activity (as defined by the ability to form a pigment with added 11-*cis*-retinal) is lost following incubation of the opsin at pH 7.5 and 25 °C for 20 days. Less than 5% is lost during the same period at 4 °C (Figure 3).

The double Cys mutation of the N2C,D282C mutant also has a dramatic stabilizing effect on the split-rhodopsin mutant SR(1−5/6−7), as is shown in Figure 4. When SR(1−5/6−7) or either the N2C or D282C mutant of SR(1−5/6−7) is bleached by exposure to light and then incubated at room temperature and pH 7.5 for 1 h, the pigments cannot be regenerated by addition of 11-*cis*-retinal (panels A−C of Figure 4, respectively). In contrast, the SR(1−5/6−7) N2C,D282C mutant fully regenerates pigment with 11-*cis*-retinal when similarly treated (Figure 4D). Also, SR(1−5/6−7) N2C,D282C opsin, like the full-length double mutant (Figure 2D), survives detergent solubilization and purification (Figure 5B), whereas the parental SR(1−5/6−7) opsin does not (Figure 5A).

We also tested the ability of the N2C,D282C double mutant to increase the stability of opsin in the context of another functional mutation in the protein. For this

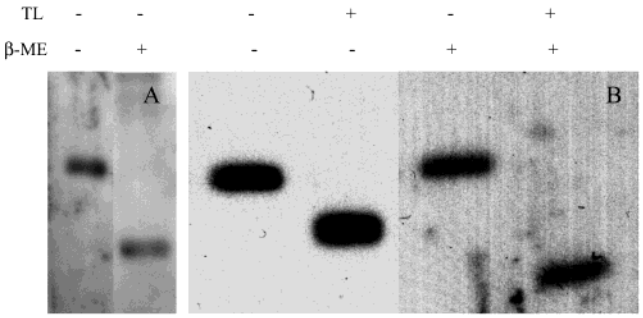

FIGURE 6: Demonstration of a disulfide bond connecting Cys2 and Cys282 in the full-length and SR(1−5/6−7) double mutants. Purification of the mutant pigments and treatment of the full-length double mutant with thermolysin (TL) were carried out as described in Experimental Procedures. Samples were then taken for Western blot analysis using Con A to probe for the N-terminal fragment of the protein. Where indicated, βME (5%, v/v) was present in the load buffer: (A) SR(1−5/6−7) N2C,D282C and (B) full-length N2C,D282C. The mobility of the protein in lane 2 of panel B is greater than that of lane 1 because thermolysin cleaves rhodopsin in the C-terminal tail (after residue 326) as well as in the 5/6 loop (at positions around residue 240) (*30*, *31*).

purpose, we chose the mutant K296G in which the Lys296 chromophore attachment site has been changed to Gly. As is shown in Figure 5C, the N2C,D282C,K296G triple mutant opsin survives detergent solubilization and purification such that when presented with the *n*-propyl Schiff base of 11-*cis*-retinal [11-*cis*-retinal itself does not form a pigment with the K296G mutant (*8*)] a pigment with spectral properties similar to those of wild-type rhodopsin is generated in high yield. Thus, the N2C,D282C double mutant does increase the stability of the opsin form of another functional mutant.

We next addressed whether the two Cys residues in the N2C,D282C double mutant form a disulfide bond. As is shown in Figure 6A, the two Cys residues do appear to form a disulfide cross-link as judged by the fact that the SR(1−5/6−7) N2C,D282C split-rhodopsin double mutant migrates on SDS−PAGE gels with the mobility of the full-length, wild-type protein in the absence of βME but with the higher mobility of the 1−5 fragment in the presence of the thiol reductant. Similarly, the thermolysin-treated, full-length N2C,D282C double mutant migrates on SDS−PAGE gels with the mobility of the full-length protein (residues 1−326; see the legend of Figure 6) in the absence of βME but with the higher mobility of the isolated N-terminal fragment in the presence of βME (Figure 6B). Therefore, we conclude that the Cys2−Cys282 disulfide bond is present in both the SR(1−5/6−7) N2C,D282C and full-length N2C,D282C double mutants.

We also note that in the presence of DTT the photobleached SR(1−5/6−7) N2C,D282C double mutant no longer contains a disulfide bond between the two Cys residues (Figure 7B) and cannot be regenerated with 11-*cis*-retinal (Figure 7A), whereas in the absence of DTT, the bond is intact (Figure 7B) and the pigment can be regenerated with added 11-*cis*-retinal (Figure 4D). While these data do not prove that the Cys2−Cys282 disulfide is responsible for the increased stability of the double mutant, they are consistent with that interpretation.

Finally, we addressed the question of whether the N2C,D282C double substitution had a significant effect on the activity of rhodopsin. For this purpose, we determined the effect of the mutation on the MII lifetime and the ability of the protein to activate transducin in response to light. As is shown in Figure 8, the N2C,D282C substitutions have very little effect on the MII lifetime, with first-order rate constants of 0.127 and 0.152 min^{-1} for decay of the wild-type and mutant MII intermediates, respectively. The mutations also have relatively little effect on the ability of rhodopsin to activate transducin in response to light. As is shown in Figure 9, the N2C,D282C double mutant activates transducin in a light-dependent manner with an initial rate that is within 20% of the value observed for wild-type rhodopsin.

DISCUSSION

We have reported here the biochemical characterization of a disulfide cross-linked mutant of rhodopsin (N2C,D282C) in which an engineered disulfide bond between Cys2 and Cys282 is formed and appears to be responsible for an increase in the thermal stability of the opsin form of the protein. The stabilizing effect of disulfide bonds on protein structure has been known for some time and is usually attributed to decreased conformational entropy in the denatured state (*26*−*29*). In the case of the N2C,D282C mutant opsin, the stability is increased to the extent that less than

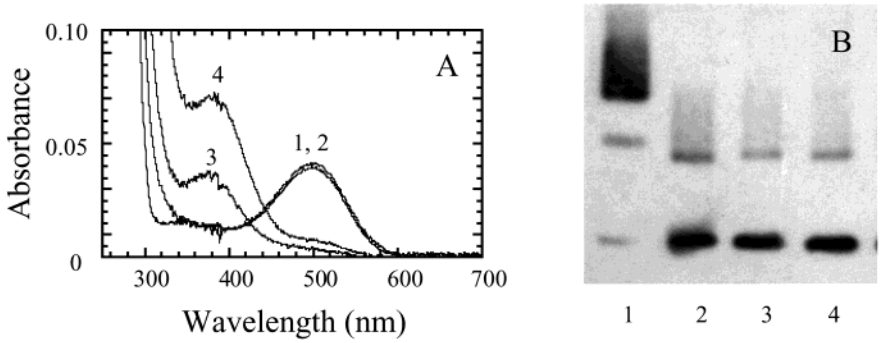

FIGURE 7: Dependence of the thermal stability on the presence of the Cys2−Cys282 disulfide bond. The split rhodopsin mutant SR(1−5/6−7) N2C,D282C was purified on the 1D4-Sepharose 4B matrix, incubated with 100 mM DTT to reduce the C2−C282 disulfide, and then tested for the ability to be reconstituted with retinal following a bleach and incubation at room temperature for 1 h: (A) absorption spectra for the pigment and (B) Western blot using 1D4 to probe for the C-terminal fragment of the protein. Samples in panel B are the same as those identified in panel A: (1) purified split rhodopsin mutant SR(1−5/6−7) N2C,D282C, (2) pigment after addition of 100 mM DTT, (3) pigment after exposure to light from a 300 W tungsten bulb filtered through a 480 nm cut-on filter for 1 min and incubation at room temperature for 1 h, and (4) pigment after addition of ~1 equiv of 11-*cis*-retinal.

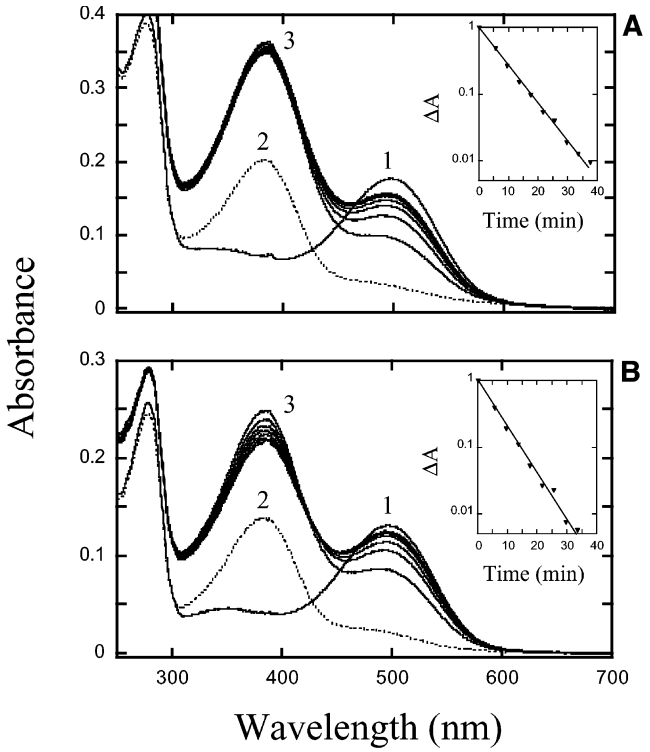

FIGURE 8: Time course of metarhodopsin II decay in wild-type rhodopsin and the N2C,D282C mutant. The rate of metarhodopsin II decay was measured by bleaching the sample and following the reconstitution of the pigment with 11-*cis*-retinal as described in Experimental Procedures: (A) wild-type rhodopsin and (B) N2C,D282C mutant. In each case, spectrum 1 is the spectrum of the purified pigment, spectrum 2 is the spectrum after exposure to light from a 300 W tungsten bulb filtered through a 480 nm cut-on filter for 1 min, and spectrum 3 is a set of spectra recorded at various times after addition of 2−3 equiv of 11-*cis*-retinal. The insets show semilogarithmic plots of the normalized change in absorbance (see Experimental Procedures) vs time after exposure to light. Solid lines in the inset were generated with first-order rate constants of 0.127 and 0.152 min^{-1} for the wild type and N2C,D282C, respectively. Some of the spectra were omitted from the main part of the figure for clarity.

50% of the active protein is lost after incubation for 20 days at pH 7.5 and 25 °C, whereas wild-type opsin does not survive the protocol for detergent solubilization and purification of the protein from transfected COS cells under these conditions. At 4 °C, less than 5% of the activity is lost after 20 days. While we have tested the effect of the mutations only on the apoprotein opsin, it is likely that increased stability may be realized in the holoprotein or rhodopsin form as well, although this remains to be explicitly demonstrated.

The C2−C282 disulfide has relatively little effect on the MII lifetime or ability of rhodopsin to activate transducin. In addition, the second-order rate constant for binding of 11-*cis*-retinal in the mutant is within a factor of 2 of that measured for the wild-type protein (G. Xie and D. D. Oprian, unpublished observations). Thus, the disulfide appears to have a minor impact on the functional properties of the protein and, for this reason, may be considered for more in-depth studies on the mechanism of Schiff base formation with the chromophore and for crystallization trials involving the recombinant protein, especially the unliganded opsin form which may be difficult to accomplish with native rhodopsin.

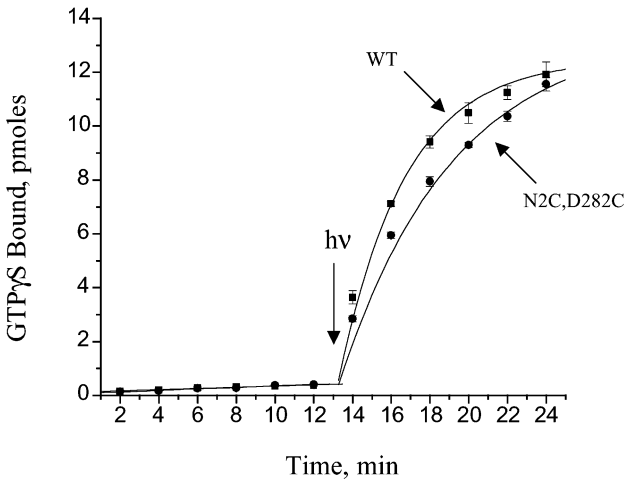

FIGURE 9: Light-dependent activation of transducin by wild-type and N2C,D282C mutant rhodopsins. Transducin assays were performed as described in Experimental Procedures. Reactions were monitored in the dark and after exposure (arrow) to light: wild-type rhodopsin (●) and N2C,D282C mutant (■). Error bars indicate the standard deviation ($n = 3$).

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