Effect of Heat and 2-Mercaptoethanol on Intracytoplasmic Membrane Polypeptides of *Rhodopseudomonas sphaeroides*

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Solubilization at 75°C of *Rhodopseudomonas sphaeroides* chromatophores in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (β-ME) resulted in the selective absence of reaction center B and C polypeptides from SDS-polyacrylamide gel electrophoresis profiles. A newly identified, chromatophore-specific polypeptide, with a mass of 35.2 kdaltons, was also missing under these conditions of chromatophore solubilization. Solubilization at 27°C in the presence of SDS and β-ME also resulted in the disappearance of these three polypeptides, but at much slower rates. Disappearance of either endogenous or exogenously supplied reaction center polypeptides B and C during SDS solubilization of whole chromatophores at either 27 or 75°C was shown to be entirely dependent upon the presence of β-ME. After chromatophore solubilization in the presence of β-ME and subsequent SDS-polyacrylamide gel electrophoresis, exogenously added reaction centers B and C could be localized in a complex of no less than 100 to 200 kdaltons. However, the precise size of the complex was influenced by the stoichiometry of the reacting components. The disappearance of the 35.2-kdalton polypeptide was neither dependent upon the presence of β-ME nor dependent upon the presence of any additional chromatophore polypeptides. The 35.2-kdalton polypeptide underwent a heat-induced oligomerization to yield several high-molecular-weight species.

The chromatophore is the isolated form of the intracytoplasmic membrane system and is the photosynthetic organelle of the facultative phototroph *Rhodopseudomonas sphaeroides* (9). This organelle is composed of approximately 50 distinct polypeptide species, of which approximately 10 comprise greater than 80% of the chromatophore protein (unpublished data). Of these 10 polypeptides, only a few have been assigned specific functions within the photosynthetic membrane. Three of these comprise the reaction center (RC) complex, an isolable subunit of the chromatophore and the site of primary events in photosynthesis (4). Clayton and Haselkorn (3) have identified the three RC polypeptides as RCA, RCB, and RCC, with approximate masses of 27, 23, and 21 kdaltons, respectively. Okamura et al. (16) have identified these same polypeptides as H, M, and L, respectively. A fourth polypeptide has been identified as the light-harvesting protein (9), and it has been suggested that it comprises more than one polypeptide species (24), although definitive proof is still lacking for *R. sphaeroides*. However, in *Rhodopseudomonas palustris*, more than one light-harvesting protein has been reported (7).

Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a powerful and efficient technique for the determination of specific polypeptide content and distribution in both RC preparations (3) and whole chromatophores (5, 9, 25). Analysis of RC preparations from the *R. sphaeroides* carotenoidless mutant, R26, by Okamura et al. (16) showed that, depending upon the buffer and SDS concentration used, the RC could be resolved into either all of its three subunits, H, M, and L, or into the H subunit and an apparent dimer composed of L and M. Using *R. sphaeroides* Y, Jolchine and Reiss-Husson (10) also obtained results suggestive of an apparent lack of dissociation of the RC. On the other hand, Clayton and Haselkorn (3) have demonstrated that the loss of RCB and RCC from either RC preparations or whole chromatophores was dependent upon the method of solubilization used before SDS-PAGE analysis of the RC preparation. Using intact chromatophores, Takemoto and Lascelles (25) also demonstrated the absence of RCB and RCC polypeptides. The addition of 4.2 M urea during solubilization prevented the apparent loss of RCB and RCC from their SDS-PAGE profiles. Similar observations with respect to the two low-molecular-weight RC polypeptides have been made with whole chromatophores from *Rhodopseudomonas cap-*
sulata (unpublished data) and Rhodospirillum rubrum (5).

These observations suggest that the apparent absence of RCB and RCC from SDS-PAGE profiles of whole chromatophores (16) is due to incomplete dissociation, under the conditions used, of the RC complex. We show that this is not the case. We further show that the apparent absence of RCB and RCC from SDS-PAGE profiles is entirely dependent upon the presence of 2-mercaptoethanol (\(\beta\)-ME) during chromatophore solubilization and that the extent of polypeptide loss is both time and temperature dependent. A high-molecular-weight complex (or complexes) containing RCB and RCC and whose formation is \(\beta\)-ME dependent has been identified, and its appearance accompanies the loss of RCB and RCC. This complex is demonstrated to contain RCB and RCC as well as one or more chromatophore-specific polypeptides.

Moreover, a comparison of SDS-PAGE profiles of chromatophores solubilized at 27 and 75°C reveals the heat-dependent absence of a major chromatophore polypeptide, heretofore unreported, with a mass of 35.2 kdaltons. We demonstrate that the heat-dependent absence of this polypeptide from SDS-PAGE profiles is in fact caused by an apparent conformational change of the polypeptide and subsequent self-oligomerization.

MATERIALS AND METHODS

Bacteria and growth conditions. R. sphaeroides wild-type strain 2.4.1 and strain PM8, a photosynthetic-minus derivative (4, 22) of 2.4.1 which does not contain RC polypeptides, were obtained from W. R. Sistrom. Photoheterotrophic growth of wild-type R. sphaeroides in medium A of Sistrom (21) was in completely filled and sealed vessels. Illumination of the cultures was provided by a bank of Lumiline incandescent lamps delivering 500 W/m² at the vessel surface. Temperature was maintained at approximately 33°C.

PM8 was also grown in medium A of Sistrom. Cultures were grown aerobically, with vigorous shaking, to a density of 10⁶ cells per ml. Chromatophore formation was induced by sparging the pregrown culture with a mixture of N₂ and air such that the dissolved oxygen concentration in the medium was maintained at 0.7 μg/ml. The dissolved O₂ concentration was continuously monitored with a Yellow Springs Instrument model 54 dissolved-oxygen meter.

\(^{35}\)S labeling of chromatophore protein was carried out in the following manner. Medium A of Sistrom was prepared, except that MgCl₂ was substituted for MgSO₄, NH₄Cl was substituted for (NH₄)₂SO₄, and Casamino Acids were omitted (LSM). K₂SO₄ and Na₂SO₄ in a molar ratio of 14:1 were then added to give a final SO₄²⁻ concentration of 0.3 mM. Medium prepared in this fashion also contained 0.06 mM sulfate due to the presence of sulfate in the added trace elements. Bacteria were precultured in LSM under photoheterotrophic conditions before subculture into fresh LSM containing 5.0 μCi of H₂\(^{35}\)SO₄ per ml. Under these conditions, 40% of the added \(^{35}\)S was recoverable as cold trichloroacetic acid precipitable material.

Chromatophore isolation. Chromatophores from freshly harvested wild-type or PM8 strains were prepared according to the method of Fraker and Kaplan (9). For isolation of \(^{35}\)S-labeled chromatophores, the Sepharose 2B purification step was omitted. Protein content was estimated by the method of Lowry et al. (13).

SDS-PAGE. Ten percent polyacrylamide-SDS slab gels (190 by 160 by 1 mm with a 15-mm stacker) were prepared according to the method of Laemmli (11). When used for analytical separations, the gels were prepared with 5-mm wells spaced 5 mm apart. The same gels were also used preparatively by using an 80-mm well. Both preparative and analytical gels were subjected to electrophoresis at 4°C with a current of 50 mA for 4 h. Twelve percent gels were also used without any change in the findings.

Chromatophores were solubilized before electrophoresis by adding 0.02 ml of chromatophore suspension, containing 5 to 10 mg of chromatophore protein per ml, to a mixture of 0.012 ml of 20% SDS and 0.005 ml of \(\beta\)-ME. Solubilization proceeded for 15 min at 75°C or for 5 min or longer at 27°C. Solubilization for 2 min at 100°C gave the same SDS-PAGE profile as did solubilization for 15 min at 75°C. A 0.02-ml amount of the solubilization mixture was placed in the appropriate sample well, and the gels were immediately subjected to electrophoresis.

After electrophoresis, the gel slabs were stained at room temperature in 0.25% Coomassie brilliant blue in 50% methanol-10% acetic acid in water. After a 3-h staining period, the gels were destained in several changes of 10% methanol-10% acetic acid in water.

Analytical gels to be radioautographed were stained and destained as described and then vacuum dried onto filter paper sheets. Dried gels were overlaid with X-ray film for 14 h. Individual polypeptides containing 20,000 dpm of \(^{35}\)S provided usable exposures within this time. For preparative gels, a different procedure was used. After electrophoresis, gel slabs were rinsed in several changes of distilled water and then wrapped in Saran Wrap. The wrapped gels were placed between two sheets of X-ray film and exposed for 18 h in the cold. Polypeptides to be isolated were localized by placing the gel slab on top of the radioautogram, and the desired regions were cut from the gel. Localization and isolation of unlabeled polypeptides from preparative gels were achieved by staining and destaining guide strips from both sides of the gel slab, which were then used as guides for removal of the desired polypeptide bands.

Polypeptides were eluted from the gel in the following manner. Gel strips were cut into 5-mm lengths and placed in dialysis bags (Sepharose 3 dialysis tubing with a molecular weight cutoff of 3,500) containing 0.5 ml of electrophoresis buffer. The entire bag was then submerged in electrophoresis buffer and subjected to electrophoresis at 12 mA for 18 h at 4°C. The electrode polarity was reversed for an additional 2 h to minimize entrainment of polypeptides in the walls of the dialysis bag. The polypeptides, now in solution, were removed from the dialysis bag and stored at 4°C in electrophoresis buffer. During the course of the extraction, a
small amount of SDS precipitated from the electrophoresis buffer. This did not, however, appear to influence the efficiency of extraction by this method. The identity and purity of all isolated polypeptides were routinely confirmed by analytical SDS-PAGE, using whole chromatophores as standards. Recoveries of isolated polypeptides ranged from 85 to 100%, and recovery of each polypeptide was directly proportional to its molecular size.

Molecular sizes of chromatophore polypeptides were estimated on analytical SDS-PAGE gels, using horse heart cytochrome c (14,800), concanavalin A (27,000), egg albumin (43,000), and catalase (57,000) as protein standards (26).

**Solubilization of whole chromatophores in the presence of exogenous polypeptides.** Each isolated 35S-labeled polypeptide was added individually to a complete, unlabeled chromatophore solubilization mixture in the following manner. Whole, unlabeled chromatophores were added to a solubilization mixture as usual. After a 1-min incubation at 27°C, an amount of a single purified 35S-labeled RC polypeptide species equivalent to the amount of that species present in the added whole chromatophores was added to the solubilization mixture. Incubation was then continued at 75°C for 15 min. After gel electrophoresis, the location of the labeled polypeptide was determined by radioautography.

**Scintillation counting.** Radioactivity in polypeptide bands from stained gels was determined by excising the band(s) of interest from dried gels and digesting it overnight in 0.5 ml of 30% hydrogen peroxide at 65°C. A 0.5-ml amount of water and 10 ml of Triton-toluene scintillation cocktail were added after digestion. Samples were counted in a Searle Analytic Isocap 300 liquid scintillation counter. Samples prepared in this fashion could be counted with 50% efficiency.

**Chemicals and reagents.** β-ME and acrylamide monomer were purchased from Eastman Kodak Co., Rochester, N.Y. The acrylamide monomer was recrystallized from hot chloroform before use. β-ME was redistilled under reduced pressure in a nitrogen atmosphere. Dithiothreitol and glutathione were purchased from Calbiochem, Los Angeles, Calif. Proteins for molecular weight standards were purchased from Sigma Chemical Co., St. Louis, Mo. Carrier-free H35SO4, with a specific activity of 25 mCi/ml was purchased from New England Nuclear Corp., Boston, Mass. Kodak No-Screen medical X-ray film was also purchased from Eastman Kodak Co. All other chemicals were of reagent grade or highest obtainable purity.

**RESULTS**

**Effect of time and temperature on SDS-PAGE profile of whole chromatophores.** Standard conditions for the solubilization and SDS-PAGE analysis of *R. sphaeroides* chromatophores are depicted in Fig. 1, lane 13. Solubilization and SDS-PAGE of chromatophores routinely lead to the selective loss of RCB and RCC (16, 25). These polypeptides constitute a significant portion of the chromatophore protein by the analysis of isolated RCs derived from whole chromatophores (3) as well as from the inclusion of urea in a solubilization mixture containing whole chromatophores (25). Because of the results of Okamura et al. (16), we considered that the routine absence of RCB and RCC resulted from the incomplete disassociation of these two polypeptides from the RC during solubilization. Urea, on the other hand, was considered to disrupt the RC complex. However, the conditions used here (Fig. 1, lane 13) were normally sufficient to totally disrupt the RC complex, as reported by Okamura et al. (16). The faintly stained band at the location of RCC in heated preparations was another polypeptide which on occasion could be resolved from RCC. Other explanations for the loss of RCB and RCC are possible. It has been demonstrated for other membrane systems that heating can result in either polypeptide self-aggregation (18) or aggregation with other polypeptides (19, 20). Alternatively, the presence of proteases in membrane preparations can lead to the alteration of standard electrophoretic profiles (1, 6, 14).

To determine if there was a heat-induced alteration of electrophoretic mobility, chromatophores were solubilized at 27 instead of 75°C. Figure 1, lane 7, represents a sample solubilized at 27°C for 5 min. It was apparent that, under these conditions, RCB and RCC were present. Further examination of the profile depicted in lane 7 revealed that solubilization at 27°C resulted in the incomplete disassociation of the chromatophore polypeptides, as seen by the increased number of bands with masses greater than 60 but less than 100 kdaltons and which were absent from lane 13. However, the profiles for polypeptides with masses below 60 kdaltons in lanes 7 and 13 were identical, with the exception that RCB, RCC, and a previously unobserved polypeptide with a mass of 35.2 kdaltons were present in the unheated sample. With the exception of the 35.2-kdalton polypeptide, the data depicted in Fig. 1, lane 13, were similar to those previously reported (9, 24, 25). Solubilization at 100°C for 2 min also caused the selective loss of these same three polypeptides.

Figure 1 also illustrates the time-dependent loss of the three polypeptides during solubilization conducted at either 75°C (lanes 8 through 14) or 27°C (lanes 1 through 7). Solubilization at 75°C led to the loss of the 35.2-kdalton, RCB, and RCC polypeptides within 2 min. Although these polypeptides appeared to be retained during solubilization at 27°C, they underwent a slow loss with prolonged incubation. The half-life for disappearance of the three polypeptides at 27°C was about 40 min. From lanes 8 through 14 we see that as RCB and RCC disappeared with time, there was at first the appearance and then...
the disappearance of material at the top of the gel. This will be discussed in greater detail later. In contrast to the observations of Okamura et al. (16), the loss of RCB and RCC was not accompanied by the appearance of an RC complex having the subunit structure ML or M2L2. The use of protease inhibitors, such as phenylmethylsulfonyl fluoride, during isolation and solubilization of the chromatophores had no effect upon the SDS-PAGE band patterns observed. Chromatophores subject to lipid extraction by the method of Folch et al. (8) still showed the heat-induced loss of RCB, RCC, and the 35.2-kdalton polypeptide. The data presented so far suggest that the loss of RCB, RCC, and the 35.2-kdalton polypeptide resulted from either a heat-induced conformational change or aggregation. To investigate this possibility further, the following set of experiments was conducted.

Interactions of isolated 35.2-kdalton, RCB, and RCC polypeptides. Radioactive 35.2-kdalton RCA, RCB, and RCC polypeptides were isolated from preparative gels of 35S-labeled chromatophores solubilized at 27°C. The radioautogram shown in Fig. 2, lanes 1, 3, 7, and 9, demonstrates the homogeneity of each of the isolated polypeptides. The 35.2-kdalton polypeptide was contaminated with several smaller polypeptides, but their presence did not affect the interpretation of the results. As indicated earlier, there was a second, minor polypeptide migrating to the same position as RCC. When isolated RC polypeptides were heated at 75°C for 15 min and rerun on SDS-PAGE, they migrated to their standard positions (Fig. 2, lanes 2, 4, and 8), indicating no heat-induced oligomerization or major conformational changes. A very small amount of each of the isolated RC polypeptides remained at the top of the gel after heating and was probably due to denaturation, since these samples were very dilute and they were not clarified by centrifugation after heating.

In contrast to the behavior of the RCB and RCC polypeptides, the 35.2-kdalton polypeptide disappeared completely upon heating and gave
rise to several new bands, as revealed by radioautography. These were significantly larger than the parent species and had masses suggestive of their being oligomers and not merely conformers of the 35.2-kdalton conformer. We have indicated, by arrows, the three largest of these bands in Fig. 2, lane 10.

When artificial mixtures of RCA, RCB, and RCC were made in all possible combinations and heated at 75°C, none of the polypeptides were displaced from their normal electrophoretic positions. In comparison with the results of Okamura et al. (16), there did not appear to be a heat-promoted complex formed between any of the RC polypeptides. It seems reasonable to conclude, by analogy to the 35.2-kdalton polypeptide, that if either RCB or RCC underwent a heat-induced oligomerization, it would have been observed.

Since the loss of RCB and RCC polypeptides occurred only when whole chromatophores were solubilized, we considered the possibility that they might interact with one or more other chromatophore-specific polypeptides in a heat-dependent reaction. The radioautogram shown in Fig. 3 demonstrates the results of an experiment where radioactive isolated polypeptides were added to and solubilized with whole, unlabeled chromatophores. As expected, the 35.2-kdalton polypeptide was distributed as a high-molecular-weight aggregate (lane 5). However, the RCB and RCC polypeptides had (in the presence of whole, unlabeled chromatophores) become associated with a high-molecular-weight complex which barely entered the top of a 10% SDS-polyacrylamide gel (lanes 1 and 2). The mass of this complex was estimated to be approximately 200 kdaltons. The amounts of RCB and RCC added to the unlabeled chromatophores were identical to the amounts used in the control experiments shown in Fig. 2. There was no radioactivity at any position on the radioautogram other than that at the top of the gel and a trace, marking the original positions of RCB and RCC. Lanes 3 and 4 represent 35S-labeled whole control chromatophores solubilized at 27 and 75°C, respectively. The disappearance of the 35.2-kdalton, RCB, and RCC polypeptides were evident from the heated sample (lane 4).

Factors affecting the formation of the high-molecular-weight complex containing RCB and RCC polypeptides. It is apparent that the disappearance of RCB and RCC from gel electrophoresis patterns of chromatophores solubilized at 75°C is not due to associations (16) with one another or protease activity but instead depends upon the presence of other, unidentified chromatophore polypeptides. Liu et al. (12) have recently demonstrated that spectrin can aggregate with several other erythrocyte ghost proteins under conditions of either high temperature or low pH. However, this aggregation is
reversible with reducing agents. Despite the presence of β-ME, a strong reducing agent, we investigated the possibility of disulfide bond formation between free sulphydryl groups of the chromatophore polypeptides. Whole chromatophores and the isolated RC polypeptides were treated with N-ethylmaleimide (NEM) as described by Phillips and Agin (17). Exogenously labeled polypeptides were added to whole, unlabeled chromatophores as described above, and mixtures were made involving either chromatophores treated with NEM together with non-NEM-treated RCB and RCC polypeptides or non-NEM-treated chromatophores with NEM-treated RCB and RCC polypeptides. The results obtained from these experiments (not presented here) were identical to those described earlier, i.e., NEM treatment of either chromatophore or RC polypeptides had no effect upon the disappearance of RCB and RCC. Additionally, if NEM-treated chromatophores were subjected to electrophoresis without added polypeptides after 75°C solubilization, the loss of the 35.2-kdalton, RCB, and RCC polypeptides still occurred in the same manner as shown in Fig. 1, lane 13. These results suggest that the interactions leading to the association of RCB and RCC polypeptides into a high-molecular-weight aggregate were not due to the formation of interchain disulfide bonds among a few select chromatophore proteins. In the case of RCB, which contains no cysteine residues (23), such a result would be anticipated.

During the NEM experiments, it was observed that the omission of β-ME led to the retention of both the RCB and RCC polypeptides after solubilization at 75°C. When chromatophores were treated at 75°C in the absence of β-ME (Fig. 4, lanes 1 and 5), the SDS-PAGE pattern for RCB and RCC was similar to that shown in Fig. 1, lane 7, for a 27°C solubilization. However, the omission of β-ME did not prevent the heat-induced modifications of the 35.2-kdalton polypeptide.

To determine if the loss of RCB and RCC polypeptides was a general consequence of the use of reductants or peculiar only to the use of β-ME, other reductants were used in place of β-ME during chromatophore solubilization. The gel shown in Fig. 4 illustrates the effects of either dithiothreitol, (lanes 3 and 8 at 75 and 27°C, respectively) or glutathione (lanes 4 and 9 at 75 and 27°C, respectively). The substitution of either of these two reductants or of dithionite or thioglycolate did not result in the absence of the RCB and RCC polypeptides during solubilization at 75°C. Moreover, the removal of β-ME by o-phenanthroline during solubilization (lanes 5 and 10 at 27 and 75°C, respectively) also prevented the loss of RCB and RCC polypeptides. Addition of 3% hydrogen peroxide to remove β-ME also prevented the loss of RCB and RCC. It is apparent that the heat-induced loss of these polypeptides is peculiarly associated with the use of β-ME. The possibility of a contaminant in the commercial grade of β-ME was ruled out by using β-ME freshly redistilled at reduced pressure under a nitrogen atmosphere. The band bending seen between adjacent lanes (for example, 5 and 6, 7 and 8, and 8 and 9) was due to the presence or absence of β-ME in certain of the solubilization mixtures. Diffusion of small molecules between neighboring lanes establishes a gradient of concentration and alters slightly the electrophoretic mobility of low-molecular-weight polypeptides and is unavoidable in an experiment such as this. Despite these aberrations, the effect of β-ME upon the loss of RCB
and RCC was clear. Although urea, when present together with β-ME at a concentration of 4.25 M during solubilization, could prevent the disappearance of RCB and RCC and the appearance of a high-molecular-weight aggregate, addition of urea after solubilization had no effect upon either the loss of RCB and RCC or the appearance of 35S associated with RCB and RCC at the top of the gel.

**Complex formation by exogenously added RCB and RCC in the presence and absence of β-ME.** If the disappearance of RC polypeptides and the concomitant appearance of an aggregate at the top of the gel were in fact, due exclusively to the presence of β-ME and independent of heating, then heating in the absence of β-ME should not result in the absence of RC polypeptides. Figure 5, lanes 1 through 4 shows unlabeled chromatophores solubilized with added [35S]RCB. Lanes 1 and 2 were solubilized at 27°C with and without β-ME, respectively. Lanes 3 and 4 were solubilized at 75°C with and without β-ME, respectively. Also shown in this figure (lanes 7 through 10) is an identical experiment using [35S]RCC. Lanes 5 and 6 represent labeled chromatophores which were solubilized at 27 and 75°C, respectively, with β-ME. As shown in lanes 3 and 9, the presence of β-ME was absolutely required during solubilization at 75°C to promote the formation of a high-molecular-weight complex involving added RCB and RCC. Moreover, the effect was specific to β-ME, since no RCB or RCC was lost upon heating in its absence (lanes 4 and 10), as might be expected if the exogenously added polypeptides were simply sequestered in a high-molecular-weight precipitate of whole or partially solubilized chromatophores formed during the 75°C incubation. Although not shown here, whole labeled chromatophores heated to 75°C in the presence or absence of β-ME yielded results identical to those obtained for the exogenously added RC polypeptides. Since exogenously added RCB and RCC were reacted with SDS before their β-ME-promoted complex formation, we assume that endogenously supplied RCB and RCC must have been reacted, to some extent, with SDS before the β-ME reaction.

**Interactions of RCB and RCC polypep-**
tides with chromatophores from mutant PM8. Chromatophores isolated from the photosynthetic (PS') mutant PM8 contain no RC polypeptides, as demonstrated by photochemical studies (4, 22). Figure 6 represents a Coo- massie brilliant blue-stained SDS-PAGE preparation of PM8 chromatophores, heated and unheated, confirming the absence of RC polypeptides as well as demonstrating reduced amounts of light-harvesting polypeptide. The 35.2-kdalton band is specific for chromatophores, and its presence in room temperature-solubilized chromatophores was apparent, as was its absence from heat-solubilized chromatophores. Thus, PM8 chromatophores are useful for determining if the formation of the high-molecular-weight complex as well as the disappearance of RCB and RCC require the simultaneous presence of both RCB and RCC, and they also might serve to indicate whether or not an as yet unidentified polypeptide(s) is necessary for the reaction.

Solubilization of PM8 chromatophores at 75°C in the presence of β-ME and exogenous, labeled RCB or RCC plus RCB resulted in the loss of either RCB or RCC and RCB, similar to that shown in Fig. 3. In the absence of β-ME, 75°C solubilization did not lead to the loss of added RC polypeptides or to the formation of the high-molecular-weight complex. These results demonstrate that either RCB or RCC alone could participate in the formation of the high-molecular-weight complex, providing that chromatophores, either wild type or mutant, were present. The complex formed by using PM8 chromatophores had a molecular weight closer to 100,000, one-half that of the complex formed...
with wild-type chromatophores.

To this point we have clearly shown that for either endogenous or exogenous RCB and RCC, their disappearance from an SDS-PAGE profile of whole chromatophores was independent of temperature, uniquely dependent upon the presence of β-ME, and dependent upon the presence of one or more other chromatophore polypeptides. Although the disappearance was independent of temperature, the rate of disappearance was clearly temperature dependent. We have further observed that the disappearance of exogenously added RCB and RCC was always accompanied by the appearance of a high-molecular-weight aggregate near the top of the gel. When wild-type chromatophores were used, the size of the aggregate was near 200,000 daltons, and when PM8 chromatophores were used, the size of the aggregate was closer to 100,000 daltons. The question then remains, can we demonstrate that the disappearance of endogenous RCB and RCC from wild-type chromatophores is accompanied by the formation of a high-molecular-weight aggregate?

Characteristics of high-molecular-weight complex and stoichiometry of RCB and RCC loss. The stoichiometry of RCB and RCC loss and their participation in the formation of the high-molecular-weight complex was investigated by using wild-type chromatophores in which all interacting components were believed to be in their correct stoichiometric ratios.

The data in Table 1, taken from the 27 and 75°C solubilizations of [35S]-labeled wild-type chromatophores shown in Fig. 7, revealed that RCB and RCC disappeared upon heating, in the presence of β-ME, in a 1:1 molar ratio. This ratio was remarkably constant and was reproducible from one experiment to the next, an it was derived from the amino acid composition data given for RCB and RCC (23). Further analysis of these data revealed that 50 to 60% of the radioactivity lost from the position of the gel normally occupied by the 35.2-kd dalton polypeptide could be accounted for by the radioactivity at the top of the gel. Since the heat-induced loss of the 35.2-kd dalton polypeptide resulted in the appearance of several high-molecular-weight oligomers (Fig. 2, lane 10), 50 to 60% recovery at the top of the gel was not unreasonable. Where, then, was the [35S]-material resulting from the loss of RCB and RCC? Comparison of the radioactivity in the stackers of the heated and unheated preparations revealed an additional 139,000 cpm in the heated sample, sufficient to account for the missing RCB and RCC plus a hypothetical polypeptide(s), “X,” with which RCB and RCC reacted. The additional label present in the stacker in the 75°C-treated preparation was not due to heat-induced denaturation of intact chromatophores, since at 75°C in the absence of β-ME very little material was normally present in this position.

If we assume, for the moment, that the disappearance of RCB and RCC was accompanied by the formation of an extremely high-molecular-weight complex, perhaps involving the hypothetical polypeptide X, how, then, do we account for the results presented in Fig. 3 and 5 and those presented for the mutant PM8, where the “complex” was observed to be in the 100,000- to 200,000-dalton range? In all of these experiments, we were following the disappearance of exogenous RCB and RCC. Under these conditions, we assume that the stoichiometry of the reactants was not optimal for the formation of the very large complex which occurs when exogenous polypeptides are absent. Only in the case of wild-type chromatophores, without the addition of exogenous polypeptides, is the stoichiometry of the reactants optimal. Such an assumption gathers support from the following observations.

As pointed out in the presentation of the results for Fig. 1, lanes 8 through 14, the gradual disappearance of RCB and RCC was accompa-

<table>
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<tr>
<th>Polypeptide</th>
<th>Solubilization at:</th>
<th>Loss/gain</th>
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<tr>
<td></td>
<td>27°C</td>
<td>75°C</td>
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<tr>
<td>RCB*</td>
<td>49,400</td>
<td>8,400</td>
</tr>
<tr>
<td>RCC*</td>
<td>43,500</td>
<td>12,700</td>
</tr>
<tr>
<td>35.2 kdaltons</td>
<td>18,900</td>
<td>4,300</td>
</tr>
<tr>
<td>Trapped in stacker</td>
<td>106,600</td>
<td>245,500</td>
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<tr>
<td>Trapped at top of gel</td>
<td>2,800</td>
<td>10,500</td>
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* Counts per minute shown for the RCB, RCC, and 35.2-kd dalton polypeptide are those found at their normal migration position after a 27°C incubation in the presence of β-ME.

* Relative molar quantities of RCB (5.85) and RCC (6.16) were determined from composition data given by Steiner et al. (23).
Finally, using the PM8 system in the same manner as described for Fig. 3, the following experiment was conducted. To a constant amount of $^{35}$S-RCC, an increasing amount of $^{35}$S-RCC was added in the presence of unlabeled PM8 chromatophores, and the amounts of $^{35}$S residing at the top of the gel and at the positions of RCB and RCC were determined. In the ratio of approximately 1 mol of RCB to 1 mol of RCC, no radioactivity appeared at the positions designated for monomeric RCB or RCC. Instead, most of the counts were at the top of the gel, and considerable material resided in the stacker. However, as the ratio of RCB to RCC increased, the aggregate became smaller, generally residing in the 100,000-dalton region of the gel. We therefore conclude that the disappearance of endogenous RCB and RCC in the presence of $\beta$-ME is accompanied by the formation of an extremely high-molecular-weight aggregate involving RCB and RCC plus one or more additional polypeptides.

DISCUSSION

RCB and RCC, whether of endogenous or exogenous origin, behave identically with respect to their absence from SDS-PAGE profiles. Their absence has an absolute requirement for $\beta$-ME, their rate of loss in the presence of $\beta$-ME is temperature dependent, urea (when present during 75°C solubilization in the presence of $\beta$-ME) can prevent their loss, destruction of $\beta$-ME in the solubilization mixture by treatment with either o-phenanthroline or $H_2O_2$ can prevent their loss, and (in the case of exogenously supplied RCs) their loss is dependent upon one or more protein components of the chromatophore.

On the other hand, when considering the loss of only exogenously supplied RCs, there is an absolute correlation between their disappearance from the gel profile and the appearance of an aggregate which contains these polypeptides and has a molecular weight of approximately 200,000, when wild-type chromatophores are used as carrier, or 100,000, when PM8 chromatophores are used as carrier. The use of chromatophores isolated from a mutant lacking RC polypeptides also demonstrates that the electrophoretic migration of either RCB or RCC can be altered in the absence of the other, thereby firmly establishing that the disappearance of these RC polypeptides is not a result of the incomplete disruption of the RC complex (Oka-mura et al. [16] or Jolchine and Reiss-Husson [10]. That the loss of RCB and RCC in the presence of PM8 chromatophores is identical to their disappearance in the presence of wild-type chromatophores during manipulation of the sol-

FIG. 7. Autoradiogram of an SDS-PAGE gel, including stacking gel of $^{35}$S-labeled chromatophores. Lanes: 1, solubilized at 27°C; 2, solubilized at 75°C. Acrylamide concentration was 12%; $\beta$-ME was present during the solubilization of both samples.
ubilization conditions lends further support both to the basic similarity of the mechanism(s) responsible for their loss and to the conclusion that one or more chromatophore-specific polypeptides play a role in RC polypeptide disappearance. We believe that the difference in the size of the aggregate when PM8, as opposed to wild-type, chromatophores are used with endogenous RCs is due to alterations in the stoichiometry of the reactants.

The major unanswered questions are the following. (i) Is the loss of endogenous RCB and RCC from wild-type chromatophores accompanied by the appearance of a high-molecular-weight aggregate? (ii) What other chromatophore polypeptides are involved and how? (iii) What is the role of β-ME?

Under what we perceive to be optimal conditions for the disappearance of endogenous RCB and RCC, we have provided evidence that not only is an aggregate formed (see Fig. 1 and 7 and Table 1), but the aggregate is capable of achieving maximal size, i.e., it just barely enters a 2% acrylamide stacker. We have also provided additional evidence that the size of the aggregate is sensitive to the correct stoichiometric relationship between the reactants by showing that we can alter the size of the aggregate as a function of reaction time or by altering the ratio of RCB to RCC. In effect, we can interfere with aggregate formation by increasing the ratio of RCB to RCC.

As to what other chromatophore polypeptides are involved and how, it is largely an open question. For several reasons, we do not believe that some component of the chromatophore is acting as an enzyme which cross-links RC polypeptides and which is β-ME activated. First, the amount of material present in the stacker (Table 1) suggests the presence of components in addition to RCB and RCC. Second, our ability to alter aggregate formation in the presence of the PM8 chromatophores by the addition of a large excess of RCB to RCC is difficult to explain if the aggregate was enzymatically formed. Finally, other reducing agents are without effect.

On the other hand, the requirement for a polypeptide X, in addition to RCB and RCC, to form the aggregate fits well with the available evidence. RCB and RCC can enter into aggregate formation in the presence of wild-type chromatophores, and there is a large excess of 35S-labeled material in the stacker (Table 1) above the amount required to account for the missing RCB and RCC. In fact, the amount of 35S label in the stacker which we suggest is due to polypeptide X would make X either a very large polypeptide or, if small, either extremely rich in sulfur or present in molar excess over RCB and RCC.

We have sliced a gel similar to that shown in Fig. 7 (data not shown) and have found that when endogenous RCB and RCC are lost, there is a concomitant loss of a large number of 35S counts from the region of the gel corresponding to a molecular weight of 14,000, which migrates at the dye front. A potential candidate for the 14-kdalton polypeptide is ferredoxin IV, identified in preparations of R. rubrum chromatophores as having a molecular weight of 14,000 and containing two Fe42+:S4 clusters (27). Although this polypeptide is most likely not a primary electron acceptor in photosynthesis, it is assumed to be bound closely to the RCs (27) and could thus be in a favorable spatial configuration to interact with the individual components of the RC during 75°C solubilization in the presence of β-ME. We can calculate that the hypothetical polypeptide X would have to possess at least 9 to 11 sulfur atoms per ml.

We cannot as yet propose a satisfactory hypothesis for the mode of action of β-ME in the formation of high-molecular-weight complexes containing RCB and RCC. However, it is possible, in fact likely, that the action of β-ME in this system is not restricted to its function as a reductant, but may in fact be dependent upon its solvent or chelation properties. Neither can we propose the nature of the interactions and the stoichiometry between the reactants, particularly with respect to the different size classes observed for the aggregate.

A search of the literature, although by no means exhaustive, has not revealed any reports of a β-ME-promoted aggregation of polypeptides. Dimer formation of protein I has been reported by Chopra et al. (2) during the preparation of outer membranes of Escherichia coli after lysozyme digestion, suggesting that extensive peptidoglycan degradation can lead to non-specific polypeptide aggregation. Such a mechanism is not likely in the case of chromatophores, as they contain no peptidoglycans. McMichael and Ou (15) have shown that β-ME, through its action as a metal-complexing agent, can influence the interconversion of two forms of a heat-modifiable protein from the outer membrane of E. coli. In this case, β-ME, as a chelator, promotes the conversion of a 28,000- to 29,000-dalton polypeptide to one of 33,000 to 35,000 daltons. The explanation for this behavior is an alteration in the conformation or charge of the polypeptide due to a variation in magnesium binding. A conformational or charge change could lead to slower migration by the form with the lower magnesium binding. The role of β-ME

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in this case is seen as removing magnesium from the protein during a 100°C-induced conformational alteration. This explanation can be ruled out in our case for two reasons: the change in molecular weight which we observe appears to be far too great to be explained by a simple conformation or charge change, and several different chelating agents do not influence the observed results.

Whether or not the effect of β-ME is unique to RCB and RCC or is more widespread, i.e., involving nonchromatophore systems, remains to be determined. However, because of the extensive use of β-ME in solubilization for SDS-PAGE systems, its action is worth investigating. If unique to chromatophores, these results may be significant with regard to the structure and function of the chromatophore, particularly the intramembrane associations of RCB and RCC.

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ADDITIONAL PROOF

Further experiments suggest very strongly that the selective loss of RCB and RCC in the presence of β-ME is due entirely to its solvent properties rather than its function as a reductant.

LITERATURE CITED


2. Chopra, I., T. G. B. Howe, and P. R. Ball. 1977. Lysoz-


12. Liu, S. H., G. Fairbanks, and J. Palek. 1977. Sponta-


15. McMichael, J. C., and J. T. Ou. 1977. Metal ion depend-


17. Phillips, D. R., and E. P. A. In. 1977. Platelet plasma mem-


23. Steiner, L. A., M. Y. Oskamura, A. D. Lopes, E. Mos-


25. Takemoto, J., and J. Lascelles. 1974. Function of mem-


27. Yoch, D. C., R. P. Carithers, and D. I. Arnon. 1977. Isolation and characterization of bound iron-sulfur proteines from bacterial photosynthetic membranes. I. Fer-