Chemotaxis in *Escherichia coli*: Associations of Protein Components†

Daniel Chelsky and F. W. Dahlquist*

ABSTRACT: Interactions between protein components of the chemotaxis mechanism in *Escherichia coli* were investigated by using the cleavable cross-linking reagent, dithiobis(succinimidyl propionate). Two methods were used to allow detection of chemotaxis-specific proteins in intact cells. The first method was to program their synthesis in the presence of [^{35}S]methionine using λ *E. coli* hybrid phages which carry the chemotaxis genes. The second method was to label endogenous methyl-accepting chemotaxis proteins (MCP's), with the methyl donor *S*-adenosyl-L-[*methyl*-^{3}H]methionine, after permeabilizing the cells with EGTA. Physical associations between proteins were analyzed, after cross-linking, by two-dimensional NaDodSO$_4$–polyacrylamide gel electrophoresis. Both labeling methods demonstrate that MCP I and MCP II exist as functional tetramers. Other proteins involved with chemotaxis were found to form dimers and higher polymers. Phage-directed products of *cheW*, *cheX*, *motA*, and *cheA* formed dimers. *CheB* and *hag* products formed multimers. A number of apparent interactions between different gene products were detected as well. Products of *cheB*, *cheW*, *cheZ*, *motA*, and *motB* were found to form complexes with other gene products. Included are results consistent with interactions between the products of *cheB* and *cheZ*.

Motile bacteria, including *Escherichia coli*, can migrate toward an increasing concentration of certain chemicals and away from others (Adler, 1975). This behavior, known as chemotaxis, involves a relatively simple form of sensory transduction in which signals received by receptors in the cytoplasmic membrane or periplasm are communicated to the flagellar apparatus. A processing mechanism exists which makes decisions that determine the swimming pattern. We have investigated the mechanism by which the signals are processed by studying the protein associations involved.

It is now clear that the presence of attractants and repellents in the medium regulates the reversible methylation of at least three proteins of the inner membrane of the bacteria. These methyl-accepting proteins (MCP's)[1] have been identified as the products of the *E. coli* genes *tsr*, *tar*, and *trg* (Silverman & Simon, 1977a; Kondoh et al., 1979). The gene products involved in the methylation and demethylation of the MCP's in *E. coli* have been identified. These include the chemotaxis-specific methyltransferase, the *cheX* product (Springer & Koshland, 1977; DeFranco et al., 1979), and a methylesterase, the *cheB* product (Stock & Koshland, 1978).

Genetic analysis has suggested additional interactions, including the association of the *cheC* and *cheZ* gene products in *E. coli* (Parkinson & Parker, 1979). The *cheC* product is thought to be associated with the flagellar basal body since the null phenotype of the gene is a loss of the flagella. The *cheZ* product may be a regulatory protein involved with the methylation reaction and is also thought to interact with the product of *cheB*, the methylesterase. To search for these and other interactions, we have used a protein-reactive cross-linking reagent to covalently trap molecules in close proximity.

This work was greatly facilitated by the use of λ *E. coli* hybrid phages carrying chemotaxis-specific genes (Silverman et al., 1976). When UV-irradiated lysogens were used as host cells, we were able to program the synthesis of chemotaxis gene products to the near exclusion of other proteins. These proteins were labeled with [^{35}S]methionine for their detection. The proteins coded by these phages have been shown to be functional by complementation of chemotaxis defective hosts (Silverman & Simon, 1977a,b).

In this paper is described a series of chemical cross-linking experiments designed to identify associations specific to the chemotaxis gene products in intact and in lysed cells. The cross-linked products observed demonstrate multimeric associations of several proteins involved in chemotaxis. The methyltransferase (*cheX*) was observed to form homologous dimers, as did the protein products of the *cheW*, *cheA*, and *motA* genes. The flagellar filament protein, the *hag* product, was observed to form a multimer as was the methylesterase (*cheB*). Several interactions between different proteins were also observed.

Materials and Methods

Chemicals. Special reagents used were [^{35}S]methionine (600–1000 Ci/mmol; Amersham), *S*-adenosyl-L-[*methyl*-^{3}H]methionine (5–15 Ci/mmol; New England Nuclear), dithiobis(succinimidyl propionate) (Pierce), sodium dodecyl sulfate (BDH Chemicals), and ultra-pure urea (Schwarz/Mann). Other electrophoresis chemicals were of electrophoresis purity.

Hybrid λ and Bacterial Strains. λ *E. coli* hybrids were from M. Silverman and M. Simon (Silverman et al., 1976). The host strain used was *E. coli* 159 lysogenized with λ *ind*$^{-}$ originally from H. Murialdo. Methylation of chemotaxis proteins was carried out in *E. coli* RP477*metF* (Parkinson & Revello, 1978) and MS5234 (from M. Simon).

Specific Radioactive Labeling of Chemotaxis Gene Products. E. coli 159λ was grown at 37 °C in AB minimal medium (Clark & Maaloe, 1967) plus 0.4% maltose plus 0.01 mg/mL thymidine from a 50-fold dilution of overnight culture to a final cell density of 3×10^{8}/mL. These were labeled according to the method of Jaskunas et al. (1975), with some modifications. Cells (5×10^{9}) were pelleted, resuspended in the same medium at 10^{9}/mL, and UV irradiated in a 10-cm glass petri plate for 15 min, 30 cm from a single Sylvannia germicidal lamp at 37 °C. This amount of irradiation was sufficient to prevent

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[1] Abbreviations used: DSP, dithiobis(succinimidyl propionate); *S*-Ado[^{3}H]Met, *S*-adenosyl-L-[*methyl*-^{3}H]methionine; MCP, methyl-accepting chemotaxis protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; NaDodSO$_4$, sodium dodecyl sulfate.

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transcription of RNA from endogenous DNA without greatly reducing the capacity of the protein synthesis machinery. After addition of magnesium sulfate to a final concentration of 20 mM, 2×10^9 cells were infected with λ hybrid phage carrying *E. coli* genes necessary for chemotaxis at a ratio of five phages per bacterial cell. These were allowed to adsorb for 15 min at 37 °C, diluted twofold, and agitated another 15 min, followed by addition of 60 μCi of [^{35}S]methionine. Typical labeling time was 45 min, followed by a 15-min chase period with 5 mM unlabeled methionine.

[^{3}H]Methyl Labeling of the Methyl-Accepting Chemotaxis Proteins. A suspension of 2×10^9 cells was treated with EGTA to permeabilize the cells to *S*-adenosyl-L-[*methyl*-^{3}H]methionine by the method of Rollins & Dahlquist (1980). Cells were washed twice in cold buffer A (10 mM Tris-HCl, pH 7.7, 10 mM $MgCl_2$, 50 mM KCl, and 1 mM EGTA) and resuspended in buffer A plus 2 M sucrose (0.5 mL/10^{10} cells) and incubated on ice for 10 min. Cold buffer A with 1 mM $CaCl_2$ substituted for EGTA was added to dilute the cells 20-fold, and the cells were pelleted and resuspended in the calcium buffer at 1×10^9 cells/mL. *S*-Adenosyl[*methyl*-^{3}H]methionine (80 Ci/mmol; New England Nuclear) was added to the treated cells (10 μCi/10^9 cells). The cells were then incubated at 22–25 °C for 40 min.

Cross-Linking Procedure. Labeled cells were pelleted twice into 20 mM $NaPO_4$, pH 8.0, plus 20 mM NaCl. DSP (0.1 mg) was dissolved into 3 μL of dimethyl sulfoxide and added to 2×10^9 cells in 1 mL of buffer. Total reaction time was 15 min at 20 °C, followed by quenching with 20 μL of 1 M hydroxylamine. Whole cells were then centrifuged and the supernatant was discarded. Alternatively, the cells were sonicated for 1 min (Branson sonifier at power level 1 with a heat systems microtip) on ice after addition of DSP. This treatment typically yielded 95% vesicles. In this case, proteins were precipitated by addition of cold trichloroacetic acid to a final concentration of 5% (w/v).

Two-Dimensional Analysis of Cross-Linked Products. A suspension of 2×10^9 cells or the precipitated proteins from these cells was dissolved in 25 μL of dissociation buffer containing 10 mM Tris–phosphate, pH 6.8, 2% $NaDodSO_4$, 8 M urea, and 0.001% Bromphenol Blue in the absence of reducing agent and heated to 70 °C for 30 min. The samples were run on $NaDodSO_4$–polyacrylamide tube gels of the Swank & Munkres (1971) type (5.5-mm i.d. × 11-cm length) buffered with 0.1 M Tris–phosphate, pH 6.8, and containing 4% acrylamide, 0.1% $NaDodSO_4$, and 8 M urea. Typically, a gel was removed from the tube and equilibrated for 1 h with 30 mL of 0.03 M Tris-HCl, pH 6.8, 0.1% $NaDodSO_4$, and 5% 2-mercaptoethanol with gentle shaking. The tube was then embedded in the top of a discontinuous Laemmli (1970) type $NaDodSO_4$–polyacrylamide slab gel using 1% agarose in the stacking gel buffer (O'Farrell, 1975). These gels were 12.5% in acrylamide and contained 0.8% bis(acrylamide). The dimensions were 0.8 mm × 14 cm × 14 cm. An identical two-dimensional gel was run with an equivalent sample which had not been exposed to the cross-linking reagent. The ^{35}S-labeled chemotaxis gene products were visualized by autoradiography using Kodak NS5T X-ray film. Tritium-labeled proteins were visualized after impregnating the gel with PPO (Bonner & Laskey, 1974) and exposing it to Kodak XR-5 X-ray film at −70 °C.

Results

Cross-Linking Products in E. coli Cells. Proteins in close association were covalently and reversibly linked by use of the cross-linking reagent dithiobis(succinimidyl propionate) (DSP).

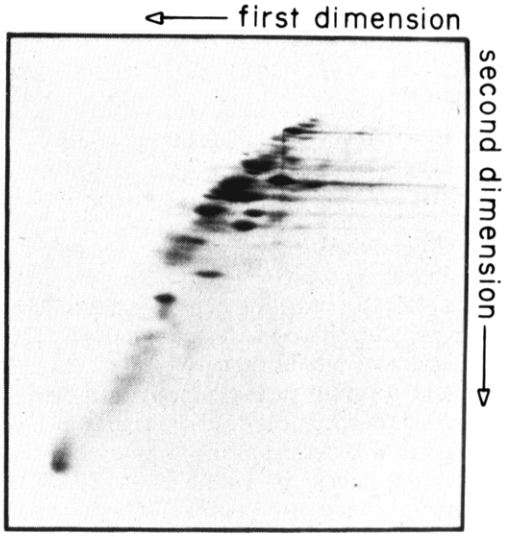

FIGURE 1: Visualization of cross-linked proteins. The cross-linking reagent DSP was added to whole cells grown on [^{35}S]methionine. An autoradiogram was then made of the two-dimensional slab gel used to analyze the products. The diagonal is composed of proteins which were not cross-linked. Spots appearing to the right of the main diagonal are representative of cross-linked products.

This bifunctional, symmetrical reagent has acylating functional groups a maximum of 11 Å apart and is bridged by a cleavable disulfide bond. The most likely stable acylated products are formed with lysine residues or other amino groups.

In order to demonstrate that DSP could be used in intact cells, proteins from *E. coli* 159λ were labeled by growth on minimal medium plus maltose and [^{35}S]methionine for four generations. Cross-linking was carried out and analyzed as described under Materials and Methods. The resulting gel was autoradiographed and is shown in Figure 1.

Molecules which ran as monomers in both dimensions formed a diagonal from the bottom left to the top right on the slab gel. Spots which appear below this diagonal result from molecules which ran at higher apparent molecular weights in the first dimension than the second and are only present when the cross-linking reagent has been used. Thus, these spots represent cross-linked products.

A dominant feature of this gel is a second diagonal which appears below and parallel to the major diagonal. This lower diagonal only appears when cross-linking reagent is present. It represents proteins which form homologous dimer cross-linked products. Many other spots appear which are not associated with either diagonal. These represent higher order homologous interactions as well as cross-links between different proteins. While many such cross-linked products can be seen, the chemotaxis proteins cannot be easily distinguished from other proteins.

Programmed Synthesis of Chemotaxis Gene Products. In order to detect the chemotaxis gene products, we used λ *E. coli* hybrid phage carrying the genes for chemotaxis and motility, constructed and characterized by Silverman & Simon (1977a,b). Expression of the *E. coli* genes carried by these phages can be detected after infection of UV-irradiated λ *ind*$^-$ lysogens. We used the host strain *E. coli* 159λ and labeled the newly synthesized proteins with [^{35}S]methionine. Up to 90% of protein synthesis, under these conditions, was programmed by the hybrid phage. The proteins were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis with subsequent autoradiography as shown in Figure 2. Each lane shows the incorporation of [^{35}S]methionine into proteins as directed by seven different λ *E. coli* hybrid phages. Each of

Table I: *E. coli* Genes Carried by the Hybrid λ *E. coli* Phage[a]

hybrid	chemotaxis genes										
	tsr	*hag*	*motA*	*motB*	*cheA*	*cheW*	*tar*	*cheX*	*cheB*	*cheY*	*cheZ*
λ*fla42*		x				x	x	x	x	x	
λ*fla42Δ5*		x				x	x				
λ*fla42Δ9*								x	x	x	
λ*fla3Δ14*						x	x	x	x	x	x
λ*fla91*	x										
λ*fla52*			x	x	x	x	x	x			
λ*fla52Δ1*			x	x	x	x		x			

[a] This table is adapted from Silverman & Simon (1977a,b).

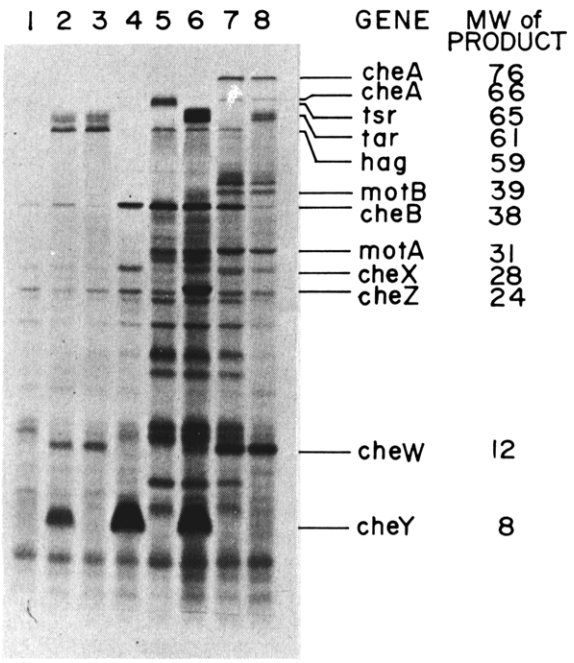

FIGURE 2: λ hybrid phage directed protein synthesis. Phage-directed proteins were labeled with [^{35}S]methionine and separated by NaDodSO$_4$–polyacrylamide gel electrophoresis (12.5% acrylamide). The autoradiogram depicts 159(λ)*flaI*$^-$ after infection with λ*fla42* (lane 1) and 159(λ)*flaI*$^+$ infected with λ*fla42*, λ*fla42Δ5*, λ*fla42Δ9*, λ*fla91*, λ*fla3Δ14*, λ*fla52Δ1*, and λ*fla52* (lanes 2–8), respectively. See Table I for a list of the *E. coli* genes carried by these phage.

the seven hybrid phages carried a different set of chemotaxis genes.

For example, lane 2 contains proteins directed by the hybrid phage λ*fla42* in the host 159λ. This phage carries the *E. coli* genes *tar*, *hag*, *cheB*, *cheX*, *cheW*, and *cheY*. The products are identified on the figure and are shown with their approximate molecular weights as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis. Lane 1 contains proteins directed by this phage when the host is 159λ*flaI*$^-$, which is defective for a regulatory function needed for expression of the *E. coli* genes carried on these phages. The low level of incorporation shown represents the background protein synthesis for this system under optimal conditions. Lanes 3–8 show the incorporation observed after infection by the phages λ*fla42Δ5*, λ*fla42Δ9*, λ*fla91*, λ*fla3Δ14*, λ*fla52Δ1*, and λ*fla52*, respectively. These phages contain various combinations of the chemotaxis genes as summarized in Table I.

Cross-Links in Hybrid Phage Infected Cells. UV-irradiated *E. coli* 159λ was infected with the hybrid phage λ*fla3Δ14*, labeled with [^{35}S]methionine and treated with the cross-linking reagent, DSP. A two-dimensional gel was used to analyze associations of the newly synthesized chemotaxis-specific proteins. An autoradiograph of this gel with a corresponding diagram, indicating the interactions, is shown in Figure 3. MCP II, the product of *tar*, displays a characteristic tetramer pattern with monomer, dimer, trimer, and tetramer spots. The products of *cheW* and *cheZ* appear to form homodimers. This determination is based on the position of the spots below the diagonal. For example, the *cheZ* product is identified as having participated in the cross-link because a spot is observed at a position corresponding to the *cheZ* monomer molecular weight (24 000) in the second, un-cross-linked, dimension and to a dimer molecular weight (48 000) in the first, cross-linked, dimension.

An additional off-diagonal spot comigrating with the *cheZ* protein in the second dimension is also apparent. This spot corresponds to a cross-linked product of approximate molecular weight 59 000 as judged from its position in the first dimension. This is indicative of an interaction between *cheZ*, 24 000, and a second component of ~35 000. The second component, if

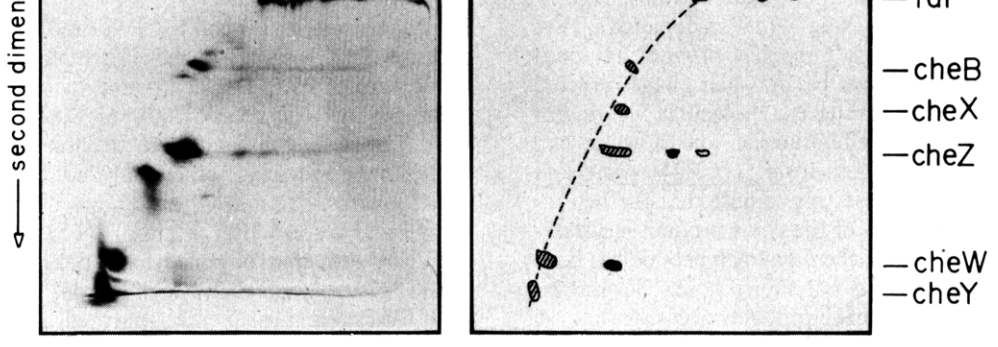

FIGURE 3: Cross-links of λ*fla3Δ14*-directed proteins. DSP was added to 159(λ) whole cells after infection with λ*fla3Δ14* and labeling with [^{35}S]methionine. The two-dimensional gel depicted in the autoradiogram (left) was run as described under Materials and Methods except that the second dimension was 10% in acrylamide. A diagram outlining the important spots (right) shows monomers as hatched, proteins involved in homologous complexes as black, and proteins which had formed heterologous complexes as white.

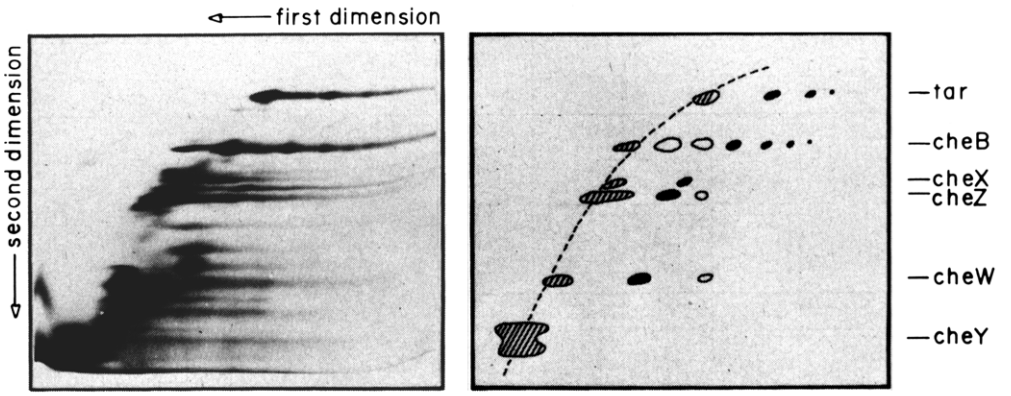

FIGURE 4: Cross-linking in a sonicated cell. DSP was added to whole cells infected with λ*fla3Δ14*, which were immediately sonicated to form vesicles. An autoradiogram of the two-dimensional gel is seen on the left. The diagram depicting the important spots (right) is described in the legend to Figure 3.

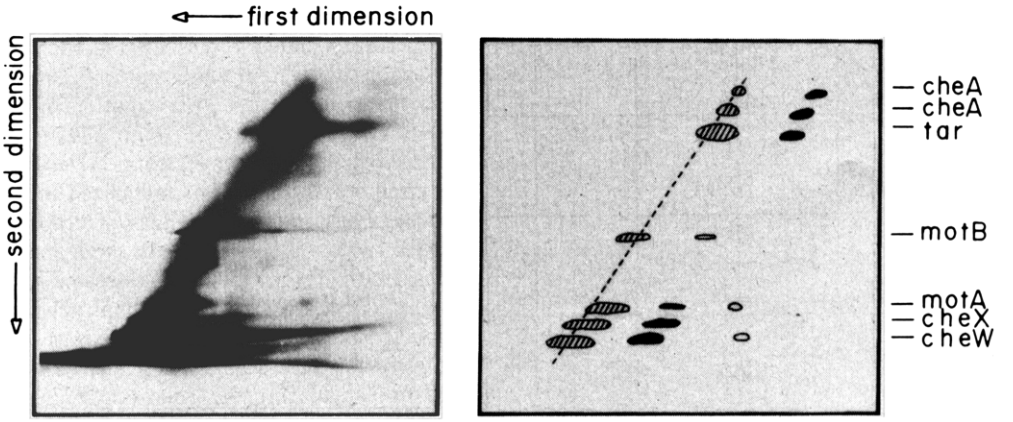

FIGURE 5: Cross-linking in a sonicated cell. DSP was added to whole cells infected with λ*fla52*, which were immediately sonicated to form vesicles. The two-dimensional gel seen (autoradiogram at left) was run as described under Material and Methods except that the first dimension was a gradient of 4–8% acrylamide and the second dimension was 5–10% acrylamide. The outlined spots on the right are described in the legend to Figure 3.

labeled, would run directly above the first spot in the second dimension at an apparent molecular weight of 35 000. Since no such spot is observed, we conclude that the 35 000-dalton protein is not directed from the phage used and must be a host protein.

Cross-linking was carried out in λ*fla3Δ14*-infected 159λ after sonication with results similar to those seen in whole cells but with additional observed interactions (Figure 4). The MCP II tetramer pattern is visible as well as off-diagonal spots corresponding to homodimers of *cheX*, *cheZ*, and *cheW* proteins. The *cheB* gene product appears here as at least a pentamer. At least four heterologous cross-link products are also visible. The *cheZ* and *cheW* proteins both form cross-linked products of ~59 000 daltons. In addition, the *cheB* protein forms a smaller complex of 52 000 daltons.

An equivalent experiment using λ*fla52* is depicted on the autoradiograph in Figure 5. The *cheA* product has been demonstrated to appear as two bands when phage-directed synthesis is employed with apparent molecular weights of 76 000 and 66 000. Apparent dimerization of these two products of *cheA*, as well as those of *tar* (MCP II), *cheX*, and *cheW*, can be seen. It is interesting to note that the heterodimer between the two forms of the *cheA* product is not observed. Weak dimerization of the *motA* polypeptide has been observed in other experiments not shown here. Several interactions between different gene products occur as well. A cross-linked product containing the *motB* protein, 39 000 daltons, is seen to have migrated at ~60 000 daltons in the first dimension, indicative of an interaction with a 21 000-dalton molecule. Similarly, the *motA* product is seen to cross-link to proteins of ~17 000 and ~49 000 daltons. No complementary radioactive spots were detected, indicating that these proteins are not coded by the specific hybrid phage used.

Cross-linking of λ*fla42*-infected cells showed that the *hag* polypeptide which is the structural subunit of the flagellum forms a dimer with some indication of higher aggregates.

Cross-Links of Endogenous Proteins. Two of the methyl-accepting chemotaxis proteins (MCP's) can be programmed by hybrid phage; *tar* (MCP II) and *tsr* (MCP I) are carried by λ*fla3Δ14* and λ*fla91*, respectively. *E. coli* 159λ was infected with each of these phages. The cells were then combined and incubated with DSP. The portion of the resulting two-dimensional gel containing the MCP region is shown in Figure 6. Both MCP I and MCP II produce tetramer patterns.

Endogenous MCP I and MCP II can be specifically labeled by using *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The cells were made permeable to this compound by an EGTA treatment developed by Rollins & Dahlquist (1980). *E. coli* RP477*metF* cells labeled in this way were treated with DSP, and the cross-linked proteins were analyzed by two-dimensional electrophoresis. An autoflourograph of the resulting gel is shown in Figure 6 (left, middle). The observed pattern is identical with that obtained from phage-directed proteins.

MS5234*tsr*$^-$ was labeled with *S*-Ado[^{3}H]Met, cross-linked with DSP, and subsequently analyzed. An autoflourograph of the resulting gel, Figure 6 (left, bottom), shows MCP II to be a tetramer in the absence of functional MCP I. A defective MCP I molecule may be present even in this case and an interaction with such a molecule cannot be ruled out.

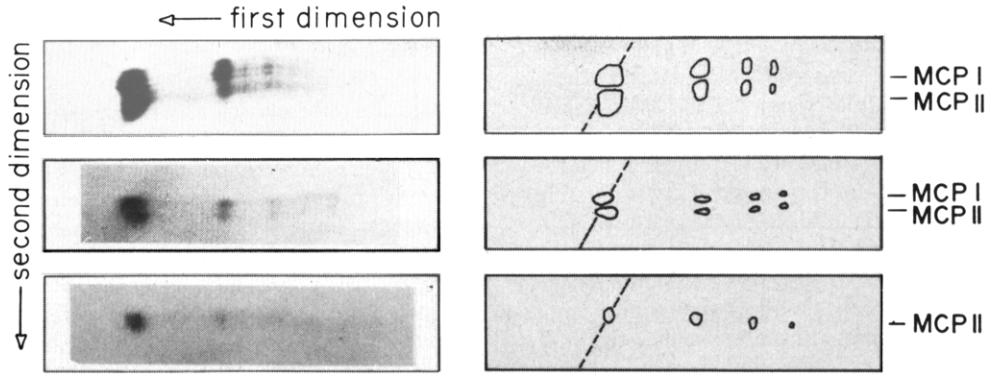

FIGURE 6: Cross-linking of phage-directed vs. endogenous MCP's. On the left are autoradiograms of portions of two-dimensional gels containing the MCP's. On the right are diagrams outlining the monomer molecules on the diagonal and dimers, trimers, and tetramers off the diagonal. Cells containing phage-directed MCP's I and II (λ*fla91* plus λ*fla3Δ14*) were cross-linked with DSP and run on the same gel (top). Endogenous *S*-Ado[^{3}H]Met-labeled MCP's from the wild-type strain RP477 (middle) as well as from MS5234*tsr*$^-$ (bottom) were cross-linked and analyzed on two-dimensional gels. The gel system is as described under Materials and Methods except that the second dimension was 6% in acrylamide to increase resolution of the 60 000-dalton proteins.

Discussion

A cleavable cross-linking reagent, dithiobis(succinimidyl propionate), has been used in whole *E. coli* cells to investigate the specific protein interactions of the chemotaxis mechanism. Techniques were employed which allowed nearly exclusive labeling of the proteins of interest despite their relatively low concentration in the cell. In this way, analysis of a complex set of proteins was made possible without any purification or major perturbation of the system. We have demonstrated the existence of a number of homologous associations as well as interactions between different gene products.

Initially, whole cells were labeled nonspecifically with [^{35}S]methionine and subjected to cross-linking and subsequent analysis. The resulting gel pattern (Figure 1) suggested that a large number of protein–protein associations existed and that they could be detected by this method. Dimers, in particular, were found to be in very high concentration relative to other interactions. Under these conditions, chemotaxis proteins could not be detected due to their low concentration in the cell. By using λ *E. coli* hybrid phage carrying the genes of interest, under conditions which prevented production of endogenous *E. coli* or phage-specific proteins (Jaskunas et al., 1975), we could specifically radioactively label chemotaxis gene products.

The cross-linked products generated by DSP which involve chemotaxis proteins are summarized in Table II. Of the gene products labeled, those of *cheW*, *cheX*, *motA*, *cheZ*, and two forms of *cheA* formed homodimers. That the *cheA* products self-associate has been predicted earlier based on extensive intracistronic complementation in that gene (Parkinson, 1977). While the two products of *cheA* apparently form dimers, as seen in Figure 5, the two off-diagonal spots do not coincide vertically as would be expected if the heterodimers were formed. Thus, although the 66 000-dalton form is derived from the 76 000-dalton form (J. S. Parkinson, personal communication), the two forms apparently do not interact. This seems reasonable since it has been shown that the larger form is predominantly membrane associated while the smaller form is located mainly in the cytoplasm (Ridgeway et al., 1977).

The structural protein of the flagella, coded by *hag*, forms a multimer. This is in agreement with electron microscopy studies which show it to be a long polymer (Abram & Koffler, 1964). The product of *cheB*, a methylesterase (Stock & Koshland, 1978) which demethylates the membrane-bound methyl-accepting chemotaxis proteins (MCP I and MCP II), also forms a multimer (Figure 4). Whether this is biologically relevant is not known, especially since the multimer form of the methylesterase is not seen in very high concentration in whole cells and is not seen at all in the whole cell two-dimensional gel shown in Figure 3.

Table II: Summary of Cross-Linked Products Observed

product of	$M_{r,app}$ × 10^{-3}	associations observed: homologous	associations observed: heterologous ($M_{r,app}$ of complex × 10^{-3})
cheA	76	dimer	
cheA	66	dimer	
cheB	38	multimer	52, 59
cheW	12	dimer	59
cheX	28	dimer	
cheY	8		
cheZ	24	dimer	59
tar (MCP II)	62	tetramer	
tsr (MCP I)	66	tetramer	
hag	54	multimer	
motA	31	dimer	48, 80
motB	39		60

MCP I and MCP II both demonstrate tetramer patterns as seen in Figure 6. Because these two proteins are very similar in function, it is reasonable that equivalent patterns were observed. In order to be sure that this pattern was representative of functional protein and not an artifact of phage-directed synthesis, we utilized a method which incorporates radioactive methyl groups into the endogenous MCP's. This was done in vivo with *S*-adenosyl-L-[*methyl*-^{3}H]methionine after permeabilizing the cells with EGTA. Thus, a direct comparison was possible between the methyl-labeled and phage-directed proteins. The results demonstrate that these two techniques do indeed give the same tetramer patterns, indicating that the cross-linked products observed in ^{35}S-labeled cells should be biologically relevent. However, because the first dimension in both gels did not distinguish between these MCP's, the possibility existed of a heteropolymer composed of both of these molecules. The probable existence of homopolymers was determined by repeating the methyl labeling in cells with a defective MCP I. The resulting pattern demonstrates that MCP II can form a tetramer in the absence of active MCP I molecules. The observed tetramers formed could represent mixtures of active MCP I and inactive MCP II subunits or they could be homopolymers.

In addition to the homologous interactions described, heterologous cross-linked products have also been observed. The methylesterase (*cheB*, 38 000 daltons) has two apparent in-

teractions in addition to its multimerization (Figure 4). These correspond to cross-linked products of ~52 000 and ~59 000 daltons. Two possible chemotaxis-specific candidates for these proteins, based on the molecular weight of the cross-linked products, are the *cheW* (12 000) and *cheZ* (24 000) proteins. Interestingly, both of these proteins are seen to form heterologous complexes of ~59 000. This would suggest possible complexes involving the *cheZ* product or the *cheW* protein dimer with the methylesterase. If the latter interaction were taking place, we would expect to observe a *cheW* monomer–*cheB* protein interaction as well. While a spot comigrating with the methylesterase at a cross-linked molecular weight of 52 000 could represent such an interaction, we would expect to see an equivalent spot directly below this, comigrating with the *cheW* protein. Since no spot is seen at that position, we believe that an interaction between the *cheW* protein and the methylesterase is not observed here.

Evidence for the interactions of the *cheZ* product with other chemotaxis proteins has been provided by Parkinson (1978) and by Parkinson & Parker (1979). The complementation properties of *cheB* and *cheZ* mutants in partial diploids and the results of reversion analysis of *cheC* and *cheZ* mutants suggest interactions of the *cheZ* product with products of *cheC* and *cheB* which are important for chemotaxis. Since the *cheC* product has not been identified, we cannot test its interaction with the *cheZ* product. Our results are certainly consistent, however, with an interaction of the *cheB* and *cheZ* gene products. This possible interaction of the methylesterase with the *cheZ* product suggests that the *cheZ* protein may act to modulate the activity of the methylesterase.

The *mot* gene products are responsible for the coupling of metabolic energy into the mechanical energy required to turn the flagellar rotor. The mechanism of this coupling remains unknown. It is interesting to note that both the *motA* (31 000 daltons) and *motB* (39 000 daltons) products cross-link to small endogenous proteins. The apparent molecular weights of these proteins are 17 000 for *motA* and 21 000 for *motB*. Since the identity of these proteins has not been determined and the molecular weight determinations are hard to pinpoint precisely, it is possible that both *mot* products are interacting with the same protein.

In addition to the above interaction, the *motA* product exists as a dimer (Figure 5). A third cross-linked product of *motA* is observed with a total molecular weight of ~80 000. The only labeled component in this cross-linked product is the *motA* protein itself. This might represent the interaction of the *motA* protein with an unlabeled protein of 50 000 molecular weight. An alternate and somewhat more likely explanation is that this represents the cross-linking of the *motA* protein dimer with the 17 000 protein which formed a cross-link to the *cheA* protein monomer.

While the identity of the proteins which are interacting with the *mot* products is unknown, they are likely to be components of the flagellar apparatus. The gene products of *motA* and *motB* are membrane bound and appear to associate with the flagellar basal body. This is indicated by the *mot* phenotype which is a paralyzed cell with intact flagella. The basal body has been shown to be composed of at least 10 polypeptides (Hilmen & Simon, 1976), some of which are in the range of those suggested by the cross-linked products seen in these experiments.

The cross-linking experiments described here provide a good deal of structural information about the proteins involved in chemotaxis and motility. Some care must be taken in the interpretation of the observed cross-links, however. For example, since we are producing radioactive proteins as a result of bacteriophage infection, one might argue that there is overproduction of these proteins relative to others. One might expect this to be particularly important for membrane-associated proteins which may aggregate in the cytoplasm or form locally high concentrations of protein in the membrane. This would lead to spurious cross-linking results. However, such arguments are unlikely to be a serious problem in our experiments. If substantial nonspecific cross-linking of this sort were to occur, one would not expect to see discrete spots in our two-dimensional $NaDodSO_4$–polyacrylamide gel electrophoretic analysis but rather a generalized smear of radioactivity running below the diagonal. This is not observed. We also see virtually no cross-linking of heterologous dimers of the various membrane-bound chemotaxis proteins as would be expected if the phage-directed synthesis of membrane proteins caused locally high concentrations of these proteins. This is further supported by experiments with endogenous MCP's. These functional proteins cross-link in an analogous manner to the phage-directed proteins. If high local concentration of protein is being created by the phage, it appears that this does not affect their associations. Thus, it appears that we are getting a relatively undistorted view of the physical associations of the proteins involved in chemotaxis and motility.

On the other hand, the fact that we do not see a cross-link between proteins does not mean that the interactions do not exist. The cross-linking depends on a fortuitous juxtaposition of reactive groups in the two proteins. In addition, certain transiently formed complexes may not give rise to a sufficiently high concentration of cross-linked products to be easily observed. A further complication arises in the case of membrane proteins. The bacterial membrane lipid phosphatidylethanolamine should react readily with the cross-linking reagent. Thus, when one end of the reagent has reacted with a lysine residue of a given membrane protein, it is very likely that the other end will react with the phosphatidylethanolamine to cross-link the protein to the lipid at the expense of protein–protein cross-links. These arguments may help to explain why we do not see cross-links of the methyltransferase (*cheX*) or the methylesterase (*cheB*) to their substrates, MCP I and MCP II.

In conclusion, we should point out that DSP and similar disulfide-linked reagents have been used successfully by others for near-neighbor analysis in isolated protein complexes (Briggs & Capaldi, 1978; Wittmann, 1976; Wang & Richards, 1974). In each case, however, only a limited number of proteins were involved which allowed for a straightforward approach to the problem. Rather than isolate a particular complex, we have resorted to cross-linking in intact viable cells where only the proteins of interest are labeled. The problems of protein isolation, as well as the artifacts associated with an isolated system, especially if it was originally membrane bound, are avoided. This approach has initially resolved a set of cross-linked products which includes many homologous interactions as well as a number of cross-links between different gene products.

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Protein–Ligand Interactions in Lumazine Protein and in *Desulfovibrio* Flavodoxins from Resonance Coherent Anti-Stokes Raman Spectra†

Richard M. Irwin,* A. J. W. G. Visser,‡ John Lee, and Lionel A. Carreira

ABSTRACT: The resonance coherent anti-Stokes Raman technique was used to obtain vibrational spectra of flavin in flavodoxins from *Desulfovibrio gigas* and *Desulfovibrio vulgaris* and of the simpler 6,7-dimethyl-8-ribityllumazine chromophore in the blue fluorescence lumazine protein from the bioluminescent bacterium *Photobacterium phosphoreum*. In the region examined, 1100–1700 cm^{-1}, the Raman spectrum of the lumazine is less crowded than that of the flavin and this facilitates assignment of observed frequencies to particular vibrational modes. Certain modes are not affected by chromophore binding to the protein, but others are changed in frequency or intensity in a way that can be rationalized by expected interactions of the chromophore with the amino acid residues of the binding site. For example, a tentative assignment of change in hydrogen bonding at N(5) is suggested as the cause of the frequency shift for the chromophore in both flavodoxins (free–bound, 1582–1572 cm^{-1}) and for C(4)=O in glucose oxidase (1359–1364 cm^{-1}) and lumazine protein (1359–1362 cm^{-1}). Shifts of the C(2)–N(3) mode in D_2O may arise from hydrogen-bonding changes at C(2)=O in lumazine protein (1284–1291 cm^{-1}), flavodoxin (1300–1280 cm^{-1}), and glucose oxidase (1297–1287 cm^{-1}). Bonding at N(3)–H may be the origin of changes in the frequency or intensity of the amide III mode in riboflavin binding protein and glucose oxidase. A stacking interaction is suggested for the change in a pyrimidine ring mode in FAD (1508 cm^{-1}) since this mode is found at 1504 cm^{-1} in 30% Me_2SO/H_2O, where the adenine and pyrimidine are unstacked. The results clearly indicate different interactions in the binding sites of those proteins studied to date.

When a small molecule binds to a protein the specific groups involved in the interaction should be identifiable by changes in their vibrational frequencies. Recently there has been a great deal of interest in the use of laser Raman spectroscopy to provide this information, and the flavins and flavoproteins have received some study, among a number of other cases. It is well-known that redox and spectroscopic properties of flavins can be markedly changed by interactions in the protein binding site. It is of interest to see if these properties are reflected by changes in the vibrational spectrum of free and bound flavin.

Coherent anti-Stokes Raman spectroscopy (CARS),[1] a nonlinear optical technique, is generally best suited for such studies because resonance enhancement (Carreira et al., 1978; Chabay et al., 1976) allows the observation of Raman signals from even highly fluorescent samples in aqueous solution, at concentrations of millimolar or less. Additionally, the analysis of observed CARS line shapes and excitation profiles can yield important structural information about the geometry of the excited state chromophore (Carreira et al., 1977a,b).

Using CARS, Dutta et al. (1977, 1978) have obtained vibrational spectra from the flavin chromophore as it exists in flavin adenine dinucleotide (FAD), bound to riboflavin binding

† From the Departments of Biochemistry and Chemistry, University of Georgia, Athens, Georgia 30602. *Received December 13, 1979.* This work was supported by the National Science Foundation, PCM 79-11064, National Institutes of Health, GM19163, U.S. Environmental Protection Agency, R-806-4300, NATO Grant 1912, and NSF CHE-7821162.

‡ Permanent address: Agricultural University, Wageningen, The Netherlands.

[1] Abbreviations used: CARS, coherent anti-Stokes Raman spectroscopy; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; LUM, 6,7-dimethyl-8-ribityllumazine; Me_2SO, dimethyl sulfoxide.

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