The Serine Receptor of Bacterial Chemotaxis Exhibits Half-Site Saturation for Serine Binding†

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ABSTRACT: Ligand binding to the serine receptor of *Escherichia coli* has been studied using isothermal titration calorimetry. Bacterial inner membranes enriched in the serine receptor (Tsr) were titrated as sonicated membrane samples and after solubilization in octyl β-D-glucopyranoside (OG) to determine the number of moles of ligand bound per mole of receptor (n), the binding constant (K_a), and the enthalpy of binding (ΔH) of serine to the receptor. The n value for serine binding to OG-solubilized Tsr protein (n = 0.5) was consistent with one molecule of serine binding to a receptor dimer, but in sonicated inner membrane samples, the n value was smaller ($n \cong 0.25$), indicating that not all of the binding sites were accessible to added serine. At 7 and 27 °C, the values for K_a and ΔH were equivalent for the membrane and OG-solubilized samples and were found to be 4.7×10^4 M^{-1} and −15 kcal/mol, and 3.6×10^4 M^{-1} and −18 kcal/mol, respectively. The influence of covalent modification at the sites of methylation on the affinity of the receptor for serine was also investigated, and found to have only a modest effect. The property of half-site saturation is suggestive of models for transmembrane signaling where the receptor subunit interactions are modulated by ligand binding.

Bacterial chemosensory systems mediate positive and negative responses to chemical gradients of attractants and repellents. In the well-studied signal transduction systems of *Escherichia coli* and *Salmonella typhimurium*, substantial progress has been made toward understanding the molecular basis of motility and chemotactic behavior. The major molecular components in the signaling pathway have been identified and their principal biochemical roles in the signal pathway have been determined [for reviews, see Parkinson (1993) and Bourret et al. (1991)]. Emphasis is now shifting toward understanding the structural organization of the molecular components, their mechanism of action at the molecular level, and a precise quantitative understanding of the stimulus–response and adaptation processes.

The initial ligand binding step in the chemosensory pathway is mediated by transmembrane receptor proteins that detect changes in the concentrations of chemoeffectors in the surrounding medium through specific ligand binding interactions. In *E. coli*, this is in part accomplished by four homologous proteins named for their principle ligands: the aspartate, serine, dipeptide, and the ribose and galactose chemoreceptors. A number of studies on the *E. coli* receptors and their analogs in *S. typhimurium* have revealed much about the organization and function of the receptors. The functional form of the receptor is a homodimer composed of two 60-kDa polypeptide chains, which are organized into three functional domains: an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic signaling domain [reviewed in Hazelbauer (1992)]. The cytoplasmic signaling domain is the physical link to the excitation pathway, modulating a phosphorylation cascade involving the CheA, CheW, CheY, and CheZ proteins [reviewed in Bourret et al. (1991)], and to the adaptation pathway, through a negative feedback loop involving reversible methylation on the receptor which is catalyzed by the methyltransferase (CheR) and the methylesterase (CheB; Springer et al., 1979).

The ligand binding pocket has been determined to lie at the interface between two receptor subunits, a property most convincingly demonstrated in the X-ray crystal structure of the ligand binding domain from the *S. typhimurium* aspartate receptor (Tar)[1] protein (Milburn et al., 1991; Yeh et al., 1993), where a single bound molecule of aspartate was found at the interface between two subunits. Remarkably, a second, nonoverlapping (symmetry-related) ligand binding site was not filled.

Ligand binding studies have delineated the specificity and affinity of the receptors (Clark & Koshland, 1979). The extent to which covalent modification of the receptor, methylation and/or amidation at specific glutamic acid residues, influences the avidity of ligand–receptor interactions has been a matter of considerable interest, since this would provide clues to the mechanism of adaptation (Hayashi & Yonekawa, 1986; Dunten & Koshland, 1991; Borkovich et al., 1992). As these experiments were carried out by different investigators using different receptors and a variety of experimental conditions, this issue has not been resolved.

In the present study, we have used ultrasensitive isothermal titration microcalorimetry to investigate the ligand binding properties of the serine receptor. We have observed that (i) the binding ratio between serine and the Tsr protein is consistent with one molecule of serine per receptor dimer, (ii) K_a and ΔH are indistinguishable in detergent-solubilized and membrane samples, and (iii) the effect of covalent modification

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[1] Abbreviations: ITC, isothermal titration calorimetry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tsr, serine receptor; Tar, aspartate receptor; IPTG, isopropyl thio-β-D-galactoside; OG, octyl β-D-glucopyranoside; PMSF, phenylmethanesulfonyl fluoride; LB, Luria broth.

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is modest in samples where the level of amidation has been varied by site-directed mutagenesis.

MATERIALS AND METHODS

Materials. Hen egg white lysozyme (catalog no. L-7773, lot no. 90H6828) and PMSF (catalog no. P7626, lot no. 43H1033) were obtained from Sigma Chemical Co. 1,10-Phenanthroline (catalog no. 1919) was purchased from Mallinckrodt Chemical Co. Isopropyl thio-β-D-galactoside (catalog no. SISO10, lot no. ZK370) was obtained from Bachem. Octyl β-D-glucopyranoside (catalog no. 494459, lot no. 910135) was the product of Calbiochem. L-Serine (catalog no. 102873, greater than 99% pure) was obtained from ICN. Reagents for BCA protein assay were obtained from Pierce. Other chemicals used were ACS reagent grade.

Bacteria and Plasmids. Escherichia coli HCB721, a strain effectively gutted of all the chemotaxis genes [relevant genotype: Δ(*tsr*)7021 *trg*::Tn*10* Δ(*cheA–cheY*)::XhoI(Tn5); Conley et al. (1989)], was obtained from H. C. Berg (Harvard University, Cambridge, MA). HCB721 has a deletion mutation that eliminates the expression of all gene products that are known to interact directly with the receptor, i.e., CheA, CheW, CheR, and CheB. The only remaining *che* gene product, CheZ, is found at 5% of the wild-type expression level in HCB721 (Conley et al., 1989). HCB721 was used in the production of Tsr-containing inner membranes since receptor protein produced in this strain will not undergo any posttranslational modification at the five known sites of deamidation and reversible methylation catalyzed by methyltransferase (CheR) and methylesterase (CheB; Rice & Dahlquist, 1991). From this host strain, the Tsr protein is thus produced with well-defined levels of covalent modification at the methylation sites, which in the wild-type Tsr receptor consists of two glutamines at positions 297 and 311 and three glutamates at positions 304, 493, and 502 (abbreviated QEQEE). Expression vectors that produce the wild-type protein, a double mutant (E304Q, E493Q) that mimics high levels of methylation by amidation at two additional sites (4Q1E), and a double mutant (Q297E, Q311E) that produces the receptor in the deamidated and demethylated state (5E) were obtained from F. W. Dahlquist (University of Oregon, Eugene, OR; Rice, 1990), and are derivatives of the pHSE5 expression vector (Muchmore et al., 1989).

Growth of Bacteria and Preparation of Membrane Vesicles. The methods employed here to prepare Tsr-containing inner membranes were similar to those described by Gegner et al. (1992) and Osborne and Munson (1974). All cultures were grown in LB (10 g/L Bacto tryptone, 5 g/L Bacto yeast extract, and 8 g/L NaCl), and were supplemented with ampicillin (150 μg/mL) when the bacteria were transformed with an inducible *tsr* plasmid. Frozen permanents of HCB721 transformed with a *tsr* plasmid (or in the case of control membranes, with no plasmid) were streaked out on LB plates, from which single colonies were used to inoculate 2-mL cultures. The turbidity of the bacterial cultures at 600 nm was used to estimate the concentration of cells; 10^9 cells/mL in a 1-cm path-length cuvette produced an apparent absorbance equal to 1 in a Cary 14 UV–vis spectrometer. Bacteria were grown at 29–30 °C for ca. 5 h in an orbital shaker at 250 rpm to a concentration of (2–4) × 10^8 cells/mL. Ten microliters from these cultures was used to inoculate 1 L of LB culturs (4 L of broth was used for each batch of preparation), which were grown under the same conditions until the cells reached a concentration of (1–2.5) × 10^8 cells/mL. Expression of Tsr was induced by the addition of 1 M IPTG to a final concentration of 0.5 mM. After 3–4 h more under the same conditions, the cells (at 7 × 10^8 to 10^9 cells/mL) were harvested by centrifugation at 4000 rpm (Sorvall HG-4L rotor) for 20 min at 4 °C. Pellets obtained from 4 L of broth were quickly resuspended in 200 mL of buffer containing 30% (w/w) sucrose/10 mM Tris-HCl, pH 8.0 at 4 °C. To this cell suspension was slowly added 2 mL of lysozyme solution (10 mg/mL in H_2O) with stirring, followed by 2 mL of 500 mM EDTA (in H_2O, pH 8.0). Cells were incubated on ice for 45–60 min. Spheroplasts were made by diluting the cells over a period of 30–60 min with 2 volumes (400 mL) of 5 mM EDTA, 1 mM 1,10-phenanthroline, and 1 mM PMSF, pH 8.0 at 4 °C, using a magnetic stirrer. (The EDTA/phenanthroline/PMSF solution was prepared immediately before use by mixing 2 mL of a 250 mM 1,10-phenanthroline/250 mM PMSF solution in absolute ethanol with 500 mL of 5 mM EDTA and adjusting the pH to 8.0.) Spheroplasts were then lysed by rapidly diluting into 4 volumes of water (2400 mL) at 4 °C. The cell lysate was centrifuged at 12 000 rpm (Sorvall GSA rotor) for 3 h. Cell pellets were immediately resuspended in 15 mL of buffer containing 30% sucrose, 10 mM Tris-HCl, and 5 mM EDTA, pH 8.0, with a tissue homogenizer. Inner membranes were separated from outer membranes using sucrose step gradients (30%, 8 mL; 45%, 8 mL; 55%, 8 mL) in six tubes, centrifuged at 28 000 rpm for 12 h and 4 °C (Beckman SW28 rotor). The majority of Tsr was found in two membranous bands, an upper and a lower band located approximately at the 30/45% and 45/55% sucrose boundaries, respectively. Tsr-containing membranes were collected from each band separately and diluted 10 times with water, or water containing 1 mM EDTA, 0.2 mM PMSF, and 0.2 mM 1,10 phenanthroline at 4 °C, and centrifuged at 12 000 rpm (Sorvall GSA rotor) and 4 °C for 4 h. The pellet from each band was resuspended in a small amount of the buffer used in titrations (0.1 M sodium phosphate, 5 mM EDTA, and 0.5 mM PMSF, pH 7.0) and was dialyzed against three changes of the same buffer (250 mL) at 4 °C. Samples were stored at 0 °C (on ice).

SDS gel electrophoresis and the BCA protein assay (Smith et al., 1985) were employed to quantify the amounts and purity of Tsr receptor in the membrane. It was found that 40–80 mg of Tsr could typically be isolated from 4 L of broth, depending on the growth conditions and the Tsr variant expressed. Of the total protein in the upper and lower bands, Tsr was (on average) 70% and 30%, respectively, as determined with a scanning densitometer. It was also found that the upper band of the membrane was better solubilized by OG than the lower band, indicating that the lower band was contaminated with outer membrane, an interpretation that was supported by the observation of prominent bands due to porins on SDS gels of this sample.

Titration Calorimetry. Titrations of Tsr receptors with L-serine were carried out on a new MicroCal MCS ultrasensitive titration calorimeter (MicroCal Inc., Northampton, MA) using Observer software for instrument control and data acquisition. Instrumentation, experimental procedures, and data analysis for a similar titration calorimeter have been described elsewhere (Wiseman et al., 1989; Brandts, et al., 1990; Lin et al., 1991). During a titration experiment, the receptor sample was thermostated in a stirred (600 rpm) reaction cell (1.343 mL), and an injection series (3 μL per injection) was carried out using a 100-μL syringe filled with a serine solution. Data points were averaged and stored at 2-s intervals. Tsr-containing vesicles (stored at 0 °C) were used for titration experiments within 2 weeks after preparation.

SDS–PAGE and titration experiments showed that the receptor was stable during this time period. Both the upper and lower bands of sucrose gradient separations were used in titration experiments. For most experiments, vesicles were further homogenized (four 15-s pulses at 60% power at 4 °C) using the intermediate tip of a sonic dismembranator (Fisher Model 300), but a few experiments were carried out without further sonication to ascertain any effect of sonication on the binding parameters. Samples of detergent-solubilized Tsr, prepared directly from aliquots of the membranous samples of receptor, were titrated subsequent to the membranous sample in a parallel experiment. Since the receptor was found to be less stable in OG, membranous samples of Tsr were solubilized by adding preweighed amounts of solid OG immediately before the titration experiments. To check for proteolysis, samples were compared by SDS–PAGE before and immediately after titration. The range of concentrations of Tsr used in titration experiments ranged from 0.02 and 0.2 mM and was determined for each sample by a combination of the BCA protein assay and SDS–PAGE (Gegner et al., 1992).

RESULTS

Tsr was isolated from *E. coli* HCB721 cells bearing the Tsr gene on an expression vector with the inducible *tac* promoter (Muchmore et al., 1989), which resulted in high expression levels of Tsr protein with the IPTG concentration (0.5 mM) that was used. Three different *tsr* expression plasmids that encoded for Tsr with different levels of receptor amidation were used. The receptors were prepared by identical methods and titrated in a similar fashion. The wild-type (QEQEE) and the amidated (4Q1E) forms of the receptor could be isolated in higher yields than the deamidated receptor (5E), which for unknown reasons was expressed at a lower level than the other forms of the receptor. Calorimetric titrations of the Tsr protein with L-serine were carried out at two temperatures (7 and 27 °C) in a 0.1 M sodium phosphate buffer, pH 7.0, containing 5 mM EDTA and 0.5 mM PMSF. For control experiments, inner membrane preparations isolated from untransformed HCB721 were also examined. Each inner membrane sample of receptor was divided into two aliquots, and solid OG was added to one aliquot immediately before titration to solubilize the receptor. Each aliquot solution was then titrated with L-serine in parallel experiments.

Figure 1a shows raw data obtained for titrations of wild-type Tsr and control membranes. The lower curve was obtained from titration of an inner membrane sample of wild-type Tsr (0.13 mM Tsr) with 2 mM serine using a schedule of 34 injections at 7 °C. The middle curve is a titration carried out between 0.13 mM Tsr solubilized in 2% OG (w/v) and 3 mM serine (36 injections) under the same experimental conditions. The sample for these two experiments was obtained from the upper band of the sucrose gradient separation, where 70% of the protein was Tsr. The uppermost curve was a control experiment consisting of 25 injections of 2 mM serine into an inner membrane sample containing no Tsr, and showed virtually no change in the size of the heat peaks throughout the entire titration.

The large exothermic peaks seen in the middle and lower curves for Tsr titrations in figure 1a are due to the binding of L-serine to the Tsr receptor. As the injections progress in each case, there is an indication that binding sites become saturated since the final peaks remain nearly invariant in size with further injections of serine. The small heat effect seen after saturation is commonly referred to as the "heat of dilution", but also includes small effects from temperature

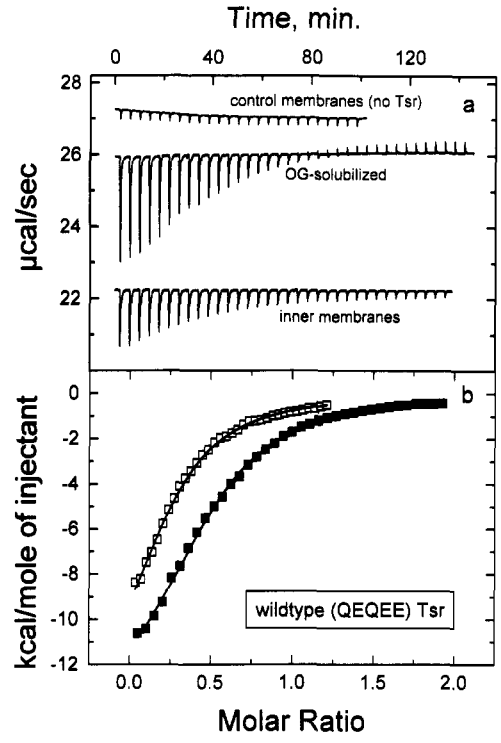

FIGURE 1: Results from titration calorimetry on the binding of L-serine to wild-type Tsr (QEQEE) at 7 °C in the absence and presence of OG (2% w/v) in buffer containing 0.1 M sodium phosphate, 5 mM EDTA, and 0.5 mM PMSF, at pH 7.0. The bottom curve in panel a shows the raw data for titration of 0.13 mM membrane receptor using injections of 2 mM serine, while the middle curve corresponds to titration of 0.13 mM OG-solubilized receptor using 3 mM serine. The upper curve is a control experiment for injections of 2 mM serine into vesicles containing no Tsr receptor. Injections for each sample were 3 µL each, spaced at 4-min intervals. Panel b shows the integrated areas for each injection, after subtracting the respective control heats, for the membrane sample (open squares) and for the OG-solubilized receptor sample (filled squares). The solid lines are the calculated best-fit curves obtained from the parameters n, K_a, and ΔH listed in Table 1.

mismatch or buffer mismatch between the liquid in the cell and the liquid being injected. It is obvious from visual inspection that the total heat (i.e., the summed area under all peaks) evolved from all injections of serine into the OG-solubilized receptor is substantially larger than for the membranous receptor, even though each of the two aliquoted samples contains the same total number of receptors. Although this might at first hand seem to suggest that ΔH of binding is larger after solubilization, it will be seen below that this arises because approximately half of the sites in the membranous samples are unavailable for binding serine. SDS–PAGE performed on each sample after titration showed no changes in the molecular weight of the receptor, indicating that no significant degradation of the sample had taken place during the titration.

The integrated heat for each injection peak in the data of Figure 1a was obtained using Origin software supplied by MicroCal, and the heat of dilution was subtracted from each data point. The normalized results are plotted in Figure 1b as kilocalories per mole of serine contained in each injection *versus* the molar ratio of serine to receptor in the reaction cell after completion of each injection. Normalized heats are shown for both the membranous receptor (open squares) and the OG-solubilized receptor (filled squares).

Data similar to those obtained at 7 °C and shown in Figure 1 were also obtained at a higher temperature of 27 °C. The integrated heats from each data set were subjected to curve-

Table 1: Thermodynamic Parameters for L-Serine Binding to the Tsr Receptor[a]

Tsr	[Tsr] (mM)	OG (%)	temp (°C)	n	K_a (M^{-1})	ΔH (kcal/mol)
wild type (QEQEE)	0.13	0	7	0.26	4.6×10^4	−15.0
	0.13	2.0	7	0.47	4.8×10^4	−15.0
	0.13	0	27	0.27	3.7×10^4	−18.5
	0.13	2.0	27	0.5	3.5×10^4	−17.5
glutamine mutant (4Q1E)	0.08	0	7	0.23	7.7×10^4	−14.1
	0.08	1.5	7	0.47	6.7×10^4	−15.0
	0.08	0	27	0.22	5.8×10^4	−18.0
	0.08	1.5	27	0.45	4.6×10^4	−18.5
unmethylated mutant (5E)	0.028	0	5.5	0.25[b]	1.0×10^5	−15.5
	0.028	1.5	5.5	0.50[b]	1.3×10^5	−16.0
	0.028	0	27	0.25[b]	7.1×10^4	−17.7
	0.028	1.5	27	0.50[b]	4.7×10^4	−18.2

[a] Buffer: 0.1 M sodium phosphate, 5 mM EDTA, and 0.5 mM PMSF, pH 7.0. [b] These values were assigned and fixed during iteration.

fitting, using a mathematical model for a single set of sites (Wiseman et al., 1989) to obtain best values for the three binding parameters: the stoichiometry n, the heat of binding ΔH, and the binding constant K_a. These parameters are listed in Table 1 for the wild-type receptor. The estimated uncertainties are approximately 10% in n and ΔH, and 50% in K_a, estimates that are based on the observed repeatability between experiments involving similar samples and experimental conditions. The solid lines in Figure 1b are the best-fit curves for the data at 7 °C resulting from the listed values of the parameters.

The data in Table 1 show that both the binding constant and the heat of binding are virtually identical for the membranous receptor and the OG-solubilized receptor. The indicated binding constant K_a for each form is 4.7×10^4 M^{-1} at 7 °C and 3.6×10^4 M^{-1} at 27 °C while ΔH is ca. −15 kcal/mol at the lower temperature and −18 kcal/mol at the higher. This strong temperature dependence of ΔH indicates a value of −150 cal °C^{-1} mol^{-1} for the ΔC_p of binding. Negative heat capacity values are consistent with a decrease in the exposure of hydrophobic side chains to water upon binding (Brandts, 1964).

The van't Hoff heat of binding can be crudely estimated from the K_a values at the two temperatures, and is −2.5 kcal/mol. This is much smaller in magnitude than the calorimetric heats of binding, and the reason for the discrepancy is not obvious. Data to be presented below on two mutant receptors also show a van't Hoff heat of much lower magnitude than the calorimetric heat.

The only significant difference in binding parameters for the membranous and OG-solubilized receptor is in the value of the stoichiometry parameter n. For the solubilized receptor, n is close to 0.5 at each temperature. This suggests that a receptor dimer acts to bind a single serine molecule, which is in agreement with the n value observed in the crystal structure of the ligand/ligand binding domain complex in the closely-related aspartate/aspartate receptor system (Milburn et al., 1991; Yeh et al., 1993). However, the value of n is only ca. 0.25 for the membranous receptor at both 7 and 27 °C. Efforts to fit the data on membranous receptors by assigning a fixed value of 0.5 to n and then floating K_a and ΔH did not produce reasonable fits; i.e., the allowable n value is highly constrained by the data. Since it seems unlikely that receptor and ligand actually interact with a different stoichiometry in the two forms of receptor, the most likely explanation for the difference in n value is that about half of the receptor molecules in the membranous preparation are unavailable for binding serine.

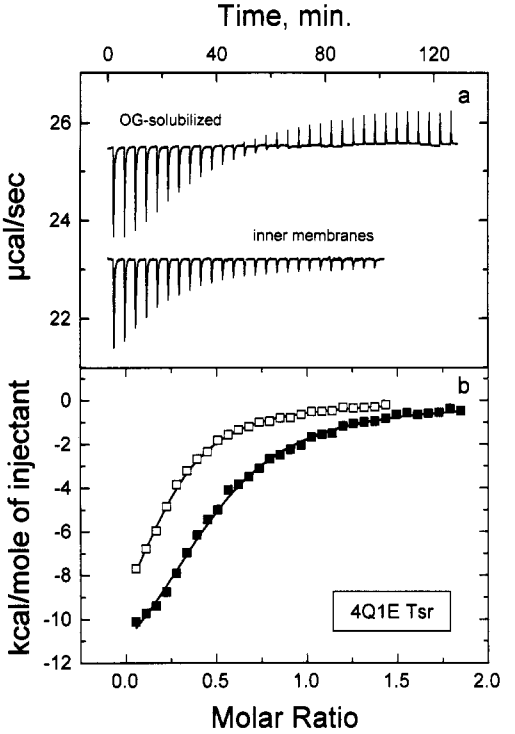

FIGURE 2: Results from titration calorimetry on the binding of L-serine to the double glutamine mutant of Tsr (1Q4E) at 7 °C in the absence and presence of OG (1.5% w/v) and in the same buffer described in Figure 1. The bottom curve in panel a shows the raw data for titration of 0.08 mM membrane receptor using injections of 2 mM serine, while the upper curve corresponds to titration of 0.08 mM OG-solubilized receptor also using 2 mM serine. Injections for each sample were 3 μL each, spaced at 4-min intervals. Panel b shows the integrated areas for each injection for the membrane sample (open squares) and for the OG-solubilized receptor sample (filled squares). The solid lines are the calculated best-fit curves obtained from the parameters n, K_a, and ΔH listed in Table 1.

This could happen if a significant fraction of the overexpressed receptors have their binding sites located within sealed vesicles which are not immediately accessible when serine is injected into the solution.

Other titration experiments (results not shown) on the wild-type receptor were carried out for samples obtained from the lower band of the sucrose gradient and for aliquot samples both with and without sonication. The K_a and ΔH values for these samples were not significantly different from those listed in Table 1. While the n value for the OG-solubilized form of the receptor was always close to 0.5, the n values for the membraneous samples varied somewhat (n ranged from 0.1 to 0.3) from batch to batch and depended on sonication. For unsonicated samples, the n values were consistently low and varied between 0.1 to 0.2. The K_a and ΔH were not strongly affected by the lower receptor concentrations taken from the lower band of the sucrose gradient. However, when titrations were performed at low receptor concentration, curve-fitting to obtain all three binding parameters is subject to larger uncertainty due to the low value of the C parameter, as discussed by Wiseman et al. (1989). In general, receptor concentrations of ca. 0.08 mM or higher are necessary if all three binding parameters are to be determined at high accuracy. At concentrations much below this, values for ΔH and K_a are highly constrained during fitting only if the n value is assigned and held fixed.

Titration of the double glutamine mutant (4Q1E) with serine was also carried out at the same two temperatures and in the same buffer. Figure 2a shows raw data at 7 °C from the

titration of both 0.08 mM membranous receptor (lower curve) and 0.08 mM detergent-solubilized (1.5% OG) receptor using 3-μL injections of 2 mM serine solution in each case. As with the wild-type receptor, it is again seen that detergent solubilization increases the number of receptors which are able to bind serine since more injections are required before saturation occurs. The blank injections, after saturation is completed, produce moderately large positive peaks for the detergent sample, likely due to a small buffer mismatch.

The raw data in Figure 2a were processed in the same way as discussed earlier in connection with Figure 1, and the integrated heats associated with each injection are shown in Figure 2b for both data sets. The best-fit parameters obtained at both temperatures are listed in Table 1 along with those discussed earlier for the wild-type receptor. Calculated titration curves for the data at 7 °C are shown as the solid lines in Figure 2b.

The binding parameters for the glutamine mutant receptor are very similar to those for the wild type at both temperatures. The calorimetric ΔH values, ca. −15 kcal at 7 °C and −18 kcal at 27 °C, are experimentally identical to those for the wild-type receptor. Indicated K values at each temperature are about 50% larger for the glutamine mutant, but this also may not be significant. Again, the n values for the mutant indicate a stoichiometry of 2 receptors for each serine binding site for the OG-solubilized form, and are also consistent with the idea that only about half of the sites are available in the membranous preparation.

Binding of serine to the unmethylated receptor (5E) was also investigated at 5.5 and 27 °C in the same buffer. Figure 3a shows the titration of 0.028 mM membranous receptor (lower curve) and 0.028 mM OG-solubilized receptor at 27 °C, using 3-μL injections of 2 mM serine. The integrated heats associated with each injection are plotted in Figure 3b. Because of the lower receptor concentration, the results from curve-fitting were less certain than for the wild-type and glutamine mutant receptor samples. When all three binding parameters are allowed to float to fit these data, the parameters are not well-confined. That is, perfectly satisfactory fits can be obtained over a fairly wide range of values for n, ΔH, and K_a since changes in each parameter can be compensated by changes in the other two in order to produce similar fit curves. On the other hand, if one of the parameters is fixed during iteration, then the other two parameters become confined. Since the n values of ca. 0.5 for the solubilized receptor and 0.25 for the membranous receptor were well-defined at the higher concentrations available for both wild-type and glutamine mutant receptors, we elected to use these as fixed parameters in order to obtain confined estimates of ΔH and K_a. The results of this data analysis are listed in Table 1, and the fit curves for the data at 27 °C are shown in Figure 3b. In view of the limitations in these data on the unmethylated mutant, the agreement of ΔH and K_a values with those of the wild type and glutamine mutant is very satisfactory.

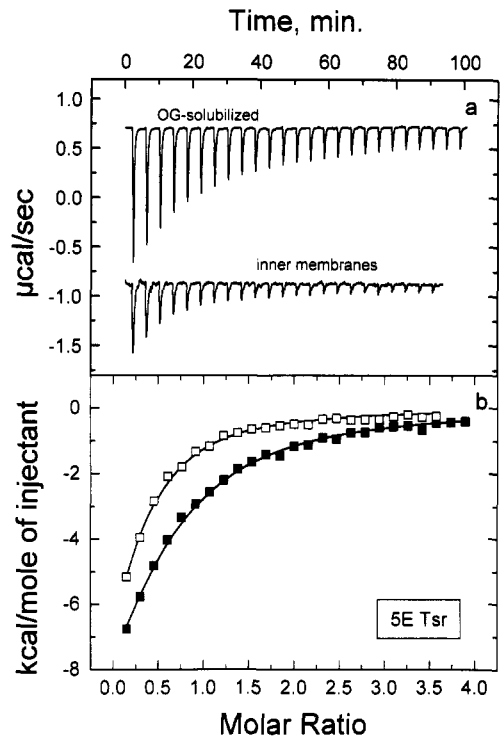

FIGURE 3: Results from titration calorimetry on the binding of L-serine to unmethylated Tsr (5E) at 27 °C in the absence and presence of OG (1.5% w/v) in the same buffer described in Figure 1. The bottom curve in panel a shows the raw data for titration of 0.028 mM membrane receptor using injections of 2 mM serine, while the upper curve is for titration of 0.028 mM OG-solubilized receptor using 2 mM serine. Injections for each sample were 3 μL each, spaced at 4-min intervals. Panel b shows the integrated areas for each injection for the membrane sample (open squares) and for the OG-solubilized receptor sample (filled squares). The solid lines are the calculated best-fit curves obtained from the parameters n, K_a, and ΔH listed in Table 1. Values of n were assigned and not determined, as explained in the text.

DISCUSSION

Effect of Temperature on Ligand Binding. The binding affinity of serine for Tsr has been studied using an ultrasensitive titration calorimeter. As a function of temperature, K_a varied from 4.7×10^4 to 3.6×10^4 M^{-1} between 7 and 27 °C for the wild-type (QEQEE) receptor. The large exothermic heat of binding (ΔH) was observed to change from −15 to −18 kcal/mol over the same temperature range, resulting in a calculated change in the heat capacity (ΔC_p) of ca. −150 cal °C^{-1} mol^{-1}. The negative value of ΔC_p for the binding reaction can be interpreted as a tightening of receptor structure and/or a net removal of hydrophobic groups from contact with water that results when serine binds to the receptor (Brandts, 1964).

One Serine Molecule Binds per Dimer of Tsr. Our results show that K_a and ΔH are nearly equivalent for the receptor in membranes and for the OG-solubilized receptor. The equivalency of K_a and ΔH for serine binding in the two buffers suggests that Tsr is predominantly dimeric under both conditions. This conclusion is supported by the observation of Milligan and Koshland (1988), who found that the closely-related *S. typhimurium* Tar was dimeric when it was solubilized in OG in the absence of ligand. However, the n values are quite different in the two samples and demonstrate that more protein is available for ligand binding in the case of the OG-solubilized receptor. A plausible explanation of the results can be made if it is assumed that all solubilized receptors are active and accessible for serine binding. These data would then suggest that one molecule of serine binds to one dimer of Tsr. This binding ratio has also been observed in the X-ray structure of the *S. typhimurium* Tar ligand binding domain, in which one molecule of aspartate was found at the interface between two subunits of the ligand binding domain (Milburn et al., 1991; Yeh et al., 1993). It has also been observed more recently in the ligand binding studies of Biemann and Koshland (1994), who reported that *E. coli* Tar exhibits half-site saturation for aspartate binding. Our data suggest that the intact serine receptor behaves in the same manner as the ligand binding domain of *S. typhimurium* Tar and the intact *E. coli* Tar, a suggestion made more plausible

in view of the homology between the three receptors. Computer simulations of the *E. coli* Tsr ligand binding pocket based on the X-ray structure of the *S. typhimurium* Tar ligand binding domain also support this interpretation (Jeffery & Koshland, 1993).

For Tsr in sonicated membrane samples, the n value (0.25) was only half that of the OG-solubilized receptor, which may indicate that half of the receptor vesicles were sealed right-side-out while the other half were sealed inside-out after sonication of the inner membrane samples. That the effective sidedness of the membrane preparations was influenced by sonication follows from the observed increase in the n value upon sonication. The lower value of n (0.1–0.2) prior to sonication is qualitatively consistent with the observation made by Gegner and co-workers (Gegner et al., 1992), who have reported that approximately 10% of the ligand binding sites are easily accessed by ligand.

Effect of Covalent Modification on Ligand Binding Parameters. One manner that adaptation in chemotaxis could be accomplished is via receptor desensitization (a lowering of its affinity for ligand), if, for example, ligand affinity depended strongly on covalent modification. In the present study, the methylation level on the receptor was mimicked by using site-directed mutagenesis to vary the number of glutamines at the sites of methylation. Isolating receptors from a strain (HCB721) deficient in the methyltransferase and the methylesterase (*cheRB*) guaranteed that the receptor had not undergone any posttranslational modification by these enzymes so that the effects of covalent modification could be studied assuming a similarity in the effects of glutamine and methyl glutamate side chains (Dunten & Koshland, 1991). The thermodynamic parameters that we have obtained for serine binding to three forms of Tsr having different levels of covalent modification, encoded genetically (deamidated, 5E; wild type, QEQEE; and amidated, 4Q1E), are not significantly different from one another as the data in Table 1 indicate. Our observations are in agreement with Dunten and Koshland (1991), who found that aspartate binding to *S. typhimurium* Tar was essentially independent of the level of covalent modification (amidation) from one extreme (all glutamates, 4E) to the other (all glutamines, 4Q). In an earlier study of ligand binding by Yonekawa and Hayashi (1986), mutant strains defective in either the methyltransferase (*cheR*), the methylesterase (*cheB*), or both (*cheRB*) were used to vary the level of covalent modification on the receptor. Employing the radioactive displacement method (Clark & Koshland, 1979), they observed that the effect of methylation on K_a was quite large and found that the affinity of Tsr for serine ($K_a \cong 1.2 \times 10^6\ M^{-1}$) obtained from membranes of a *cheR* mutant, i.e., deficient in the methyltransferase but wild type for the methylesterase, was ca. 100 times higher than the amidated and methylated form of Tsr in membranes prepared from a *cheB* mutant (deficient in the methylesterase, but wild type for the methyltransferase). A qualitatively similar effect was observed for Tar (~10-fold difference in affinity between the two extremes). These data led to the conclusion that receptor desensitization (and thus adaptation in chemotaxis) was mediated by covalent modification in the cytoplasmic domain. Borkovich et al. (1992) have also studied the effect of covalent modification on the aspartate affinity with *E. coli* Tar. They were able to prepare homogeneously methylated forms of the receptor, and found that the binding affinity of aspartate differed by a factor of 5 between the unmethylated (4E, $K_a = 2.8 \times 10^5\ M^{-1}$) and tetramethylated (4methyl-E, $K_a = 5.4 \times 10^4\ M^{-1}$) forms of Tar (Borkovich et al., 1992), in qualitative agreement with the results of Hayashi and Yonekawa (1986).

The rather significant effect of covalent modification on the binding constant that was observed by Yonekawa and Yayashi (1986) and not observed in more recent studies may be partly a result of sample preparation techniques. (1) In our study and the other more recent reports of ligand binding (Dunten & Koshland, 1991; Borkovich et al., 1992), the receptors were overproduced (perhaps as much as 50–100-fold) using expression vectors. Only in the study by Yonekawa and Hayashi (1986) were the receptor concentrations in the membrane at physiological levels. (2) The strain of *E. coli* used in our preparation of Tsr receptor was devoid of all the components (or proteins) of the chemotaxis apparatus that are known to interact with the receptor. The bacterial strains used by Yonekawa and Hayashi (1986), apart from defects in *cheR* and/or *cheB*, still possessed the other chemotaxis genes, notably *cheW* and *cheA*, whose gene products are known to interact with the receptor (Gegner et al., 1992). It is possible that through these interactions the effects of methylation on ligand binding affinity may be exerted. (3) Only the present study has used samples prepared with sucrose gradients to separate the outer from the inner membranes.

It would be instructive to compare the current tiration calorimetry results with earlier binding data for the Tsr receptor, and for the aspartate receptor which has been investigated more thoroughly. Yet the differences in the methods of preparation and characterization preclude a critical comparison of results. Investigations where (i) the different methods of sample preparation are compared using a single experimental technique and where (ii) different experimental techniques are compared using a uniformly prepared sample, e.g., the same type of receptor, the same receptor density, and the same level of covalent modification, would address the discrepancies that have been observed in the various studies. Nevertheless, it is worth pointing out that our most precisely determined value of K_a for Tsr is roughly 5 times smaller than the value reported by Clarke and Koshland ($2 \times 10^5\ M^{-1}$) at 0 °C (Clarke & Koshland, 1979).

In summary, the present titration data and the previous binding studies from other laboratories (except that of Yonekawa and Hayashi) suggest that covalent modification of the cytoplasmic domain on Tsr and Tar does not significantly affect ligand binding. This might imply that covalent modification in the cytoplasmic domain has little or no effect on the structure of the periplasmic binding domain. Since the distance between the binding site and the covalent modifying site is greater than 100 Å, it is perhaps not unexpected that domain–domain interactions across the membrane are weak. It should be emphasized that our study was carried out under nonphysiological conditions, most notably at an elevated (2-d) concentration of receptor, and in the absence of the other chemotaxis proteins that function in a complex with the receptor *in vitro* (Gegner et al., 1992; Schuster et al., 1993). It remains to be seen if the presence of the cytoplasmic chemotaxis proteins (CheA, CheB, CheR, or CheW) will alter more profoundly the affinity of the ligand to the receptor as a function of covalent modification.

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