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Solubilization of a vectorial transmembrane receptor in functional form: Aspartate receptor of chemotaxis

(sensory transduction/adaptation/membranes/reconstitution)

ELENA BOGONEZ AND DANIEL E. KOSHLAND, JR.

Department of Biochemistry, University of California, Berkeley, CA 94720

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ABSTRACT **The aspartate receptor, an integral membrane protein in the bacterial chemosensory system, has been solubilized in functional form by a combination of detergent, phospholipid, and glycerol. The conformation of the solubilized receptor is the same as that of the protein *in vivo*, as indicated by aspartate binding, rates of methyl esterification, and quantitative correlation of stimulus with this covalent modification. Studying the functional solubilized receptor in a homogeneous solution avoids many difficulties associated with an *in vivo* or a vesicle-reconstituted receptor. The technique of adding lipids, detergent, and glycerol to solubilize the protein in active form appears to be generally applicable.**

The topology and membrane solubility of receptors pose special problems to the investigator interested in clarifying their mechanisms (see Fig. 1). Receptors are usually integral membrane proteins that are vectorially inserted into a specific membrane. To restore function, the purified receptor protein, which often tends to aggregate or precipitate, must be reconstituted into an artificial membrane (1, 2). Even if the receptor is active under these conditions, new problems arise. During reconstitution into lipid vesicles, the receptor generally adopts a random orientation, so that vectorial properties are obscured and high backgrounds are observed in functional studies.

A solubilized functional receptor that has properties identical to those in the natural membrane can greatly accelerate many studies. Components of the reaction can be added in various permutations and the kinetics of the response can be measured as in a soluble enzymatic system. A prerequisite for success is the availability of assays which demonstrate that the solubilized receptor is an accurate representation of the *in vivo* receptor, and in a number of cases recovery of some, if not all, of the measurable properties have been obtained (3–9).

The aspartate receptor in bacterial chemotaxis is a particularly felicitous choice for a test of "native" conformation based on several discriminating criteria. The receptor is known to bind aspartate on the outside of the membrane (10, 11) and to be esterified with methyl groups at several glutamate residues on the cytoplasmic side (12–15). In nonstimulated cells the basal level of methylation is maintained through a steady state of methylation and demethylation catalyzed by a methyltransferase and a methylesterase (16–18). In the presence of attractants the methylation of the receptor increases to a new steady-state level, a phenomenon that we shall refer to as the "coupling" of stimulus to methylation level (19, 20). The receptor has been reconstituted in lipid vesicles (11). Thus, the aspartate receptor has several properties—aspartate binding, methylation ability, and coupling—that can be used to test for the native conformation.

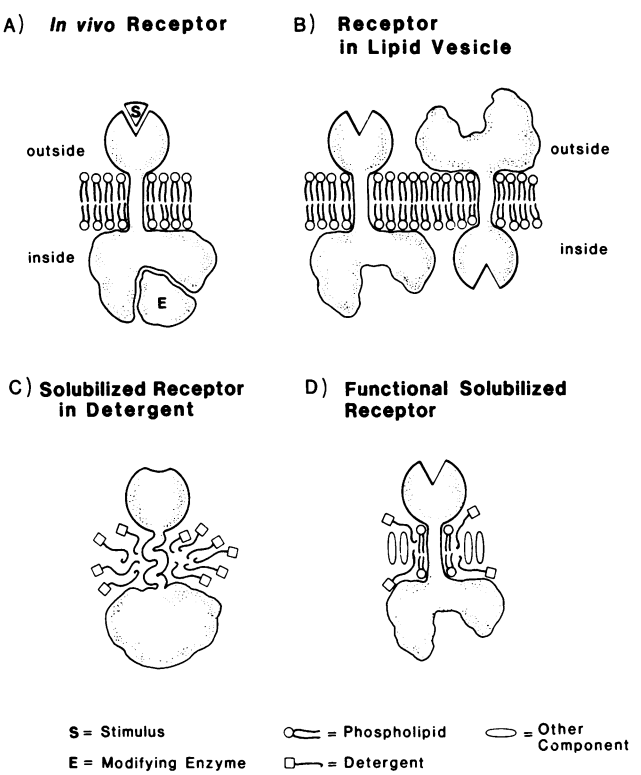

FIG. 1. Representation of a transmembrane receptor molecule in various situations. (*A*) *In vivo* receptor imbedded in phospholipid bilayer and binding stimulus and covalent modifying enzyme. (*B*) Receptor reconstituted in artificial vesicle has correct *in vivo* conformation but 50% of receptors are oriented one way and 50% are oriented the other. (*C*) Receptor solubilized in detergent has soluble portions exposed but lacks correct conformation for reaction. (*D*) Receptor in "miscibility mixture" of detergent, phospholipids, and glycerol has correct conformation and both "soluble" regions are exposed.

Forming a solution of a functional receptor is realistic if current ideas of proteins and membranes are considered. In Fig. 1 are shown aspects of the aspartate-binding protein, including potential lipid and detergent interactions. The aspartate receptor contains highly hydrophobic sequences, which are proposed to span the lipid bilayer, flanked by sequences that have the composition of soluble proteins (21, 22). According to (*i*) theories of Helenius and Simons (23) and Tanford and Reynolds (24) among others, and (*ii*) experiments by Clarke (25) and Bordier (26), nonionic detergents should bind to the lipid-binding parts of the protein and not to the "soluble protein" regions. Moreover, detergent should

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Abbreviations: OcGlc, octyl β-D-glucoside; Mes, 2-(*N*-morpholino)ethane sulfonate; AdoMet, *S*-adenosylmethionine.

not bind to the methylating or demethylating enzymes, since they are soluble proteins. As shown in Fig. 1, the detergent could solubilize the protein while leaving both the aspartate-binding region and the methylatable regions free to react. However, the solubilized protein is often inactive; thus the goal is to find some combination of detergents and other additives that will solubilize the receptor in an *in vivo* conformation (27–31).

In the studies reported below, the aspartate receptor from *Salmonella typhimurium* has been dissolved in aqueous solution by means of detergent and then systematically treated with additives to restore function. Receptor activity was tested by aspartate binding, kinetics of the methylation and demethylation, and the enhancing power of aspartate on the covalent modification level. The results suggest that the receptor has been solubilized in a functional form with a conformation similar to that *in vivo*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *Escherichia coli* strains RP4080 (*cheR*$^-$), RP4372 (*tar*$^-$, *tsr*$^-$, *tap*$^-$), and RP1091 (an *E. coli* mutant with genes from *cheA* through *cheZ* deleted) were obtained from J. S. Parkinson, University of Utah (32). The plasmids pWK35 (which contains the *S. typhimurium* aspartate receptor gene, *tar*$_s^+$, in which the subscript s refers to the *Salmonella* gene), pGK3 (*tar*$_s^+$, *cheR*$_s^+$), pGK2 (*tar*$_s^+$, *cheR*$_s^+$, *cheB*$_s^+$), and pDK1 (*tar*$_s^+$, *cheR*$_s^+$, *cheB*$_s^+$, *cheY*$_s^+$, *cheZ*$_s^+$) were previously constructed in our laboratory (15, 33–35). Bacterial membranes containing unmethylated aspartate receptor were obtained from the *E. coli cheR*$^-$ strain RP4080, transformed with plasmid pWK35 (35). Cytosolic extracts enriched in methyltransferase were obtained from strains RP4372 and RP1091 transformed with plasmids pGK2, pGK3, or pDK1.

Growth of Cells and Preparation of Plasma Membranes and Cytosolic Extracts. Bacteria were grown and membrane and cytosolic extracts were isolated as previously described (36) with the following modifications. Cells were disrupted by sonication in 5 mM phenanthroline/0.2 mM *p*-hydroxymercuribenzoate/1 mM phenylmethylsulfonyl fluoride/50 mM sodium phosphate, pH 7.0. Intact cells and large fragments were removed by centrifugation at 12,000 × *g* for 20 min. Membrane pellets were resuspended in 2 M KCl/5 mM EDTA/1 mM phenanthroline/0.5 mM phenylmethylsulfonyl fluoride/0.1 mM *p*-hydroxymercuribenzoate/20 mM sodium phosphate, pH 7.0, spun at 100,000 × *g*, and then washed again in the same buffer without *p*-hydroxymercuribenzoate. These high-salt-washed membranes were finally resuspended in 5 mM EDTA/5 mM phenanthroline/1 mM phenylmethylsulfonyl fluoride/100 mM sodium phosphate, pH 7.0, and spun as before. Membrane pellets were frozen and stored at −20°C.

Cytosolic fractions obtained in the first high-speed centrifugation of crude extracts were dialyzed against 5 mM EDTA/50 mM 2-(*N*-morpholino)ethanesulfonate (Mes), pH 6.0, divided into aliquots, and stored at −20°C, at a protein concentration of 8–12 mg/ml.

Preparation of Extracts with Octyl β-D-Glucoside (OcGlc). Bacterial membranes prepared as described above were resuspended at a protein concentration of 5 mg/ml in buffer containing 1 mM EDTA, 1 mM phenanthroline, 0.2 mM phenylmethylsulfonyl fluoride, and 25 mM Mes, pH 6.0. OcGlc was added at a final concentration of 1% (wt/vol), and the mixture was incubated at 4°C for 30 min. After centrifugation at 100,000 × *g* for 2 hr at 4°C to sediment detergent-insoluble material, supernatants, referred to as OcGlc extracts, were divided into aliquots and stored at −20°C.

Incubation of OcGlc Extracts with Phospholipids. Standard incubation mixtures contained the following: ½ vol of OcGlc extract (obtained as described above, in 1% OcGlc), 40% (wt/vol) glycerol, *E. coli* phospholipids at 1.8 mg/ml, 0.5 mM EDTA, 0.5 mM phenanthroline, 0.1 mM phenylmethylsulfonyl fluoride, and 12.5 mM Mes, pH 6.0. Mixtures were incubated for 1 hr at 22°C before aspartate-binding or methylesterification assays. Storage for 1 month at −20°C did not result in apparent loss of reconstituted activity. Before addition to OcGlc extracts, phospholipids were sonicated for 1 min in a bath sonicator under N_2 atmosphere.

Sites of methyl esterification *in vitro* of solubilized aspartate receptor were identified as described earlier (15).

RESULTS

Solubilization of the Receptor with Retention of Methylation Capacity and Aspartate Binding. To test whether the receptor could be solubilized in a functional form, it was first tested for its methylation capacity. Early attempts using nonionic detergents [OcGlc, octanoyl-*N*-methylglucamide (Mega 8), or decanoyl-*N*-methylglucamide (Mega 10)] or zwitterionic detergents [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (Chaps), *N*-dodecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-12), or *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate (Zwittergent 3-14)] gave soluble receptors but none were methylated by cytosolic extracts enriched in methyltransferase. Addition of an *E. coli* phospholipid preparation to an OcGlc-solubilized receptor produced no significant methylation even at phospholipid concentrations of 3 mg/ml. When glycerol as well as phospholipids were added to the OcGlc-treated receptor, methylation occurred, and it increased with increases in phospholipid and glycerol levels (Fig. 2). At 40% glycerol in the reconstitution step (20% in the methylation assay) concentrations of phospholipids higher than 1 mg/ml gave a methylation level 2 times that of receptors bound to vesicle membranes.

A second test of the functionality of the receptor is to measure ligand binding. The dissociation constant for [^{14}C]aspartic acid in the presence of phospholipids and glycerol was determined by a filter binding assay for soluble proteins. The value derived from a double-reciprocal plot of the binding curve was 3×10^{-6} M, which is similar to the values previously reported for the native (6 μM) (10) and purified (3 μM) receptor (11).

Methylation of the Solubilized Receptor. A third criterion of the conformational state of the receptor is the ability of added aspartate to alter the methylation level. As shown in Fig. 3, aspartate added at the beginning or after 70 min of the methylation reaction produced a 7-fold stimulation of the methylation rate (Fig. 3). Nonspecific activation of methyltransferase was ruled out since serine, which is known to act through a different receptor protein, did not stimulate methylation rate.

An inhibition of basal demethylation was also observed (Fig. 3). After 70 min, a 50-fold excess of unlabeled AdoMet, with or without 1 mM aspartate, was added to aliquots of the media. In the absence of aspartate the decrease in [^{3}H]methyl groups present in proteins gave a demethylation rate constant of 1.69 hr^{-1}. When aspartate was added at the time of the chase with unlabeled AdoMet, this loss of [^{3}H]methyl groups was inhibited. This agrees with *in vivo* observations of attractant inhibition of demethylation (38).

In Fig. 4 the relative stimulation of methylation rates at various pH values are shown. As can be seen, there is a maximal difference in rates at pH 5.5 and 7.0 but significant differences at other pH values. In the *in vivo* system a difference in rates in the presence and absence of aspartate of 1.6- to 2-fold is seen when the cell is approximately pH 7.5 with an ionic strength of 150–200 mM. Thus, the methylation

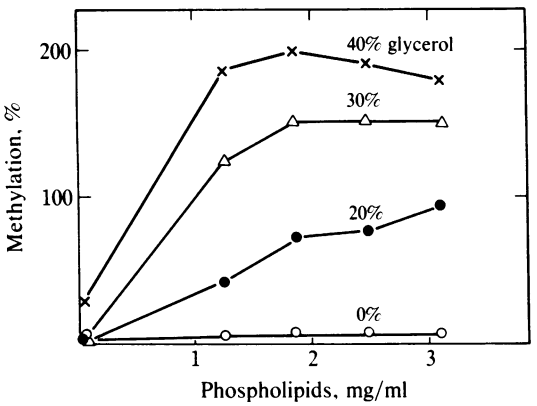

FIG. 2. Effect of glycerol and phospholipids from *E. coli* on the methylation of detergent-solubilized aspartate receptor. Receptor-enriched membranes from *E. coli* RP4080 transformed with plasmid pWK35 were solubilized with OcGlc and the extracts were then incubated for 1 hr at 22°C with increasing amounts of *E. coli* phospholipids at the four indicated concentrations (wt/vol) of glycerol. Reaction media contained 50 μl of standard OcGlc extract/phospholipids/glycerol mixtures and 50 μl of methylation medium [200 μM *S*-adenosyl[*methyl*-^{3}H]methionine (Ado[^{3}H]Met; 350 cpm/pmol), 25 μl of cytosolic extracts enriched in methyltransferase, 2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM phenanthroline, 50 mM Mes, pH 7.0 at 32°C]. At 90 min, 10-μl samples were removed and pipetted onto squares (1 cm) of Whatman 3MM filter paper, which were dropped immediately into cold 10% trichloroacetic acid. Filter papers were extensively washed with 10% trichloroacetic acid, then 100% methanol, and allowed to dry at room temperature. Radioactivity present in carboxymethyl groups was determined by means of a diffusion assay previously described (15, 37), with an efficiency of 80 ± 10%. A background value obtained from the methyl groups present in cytosolic extracts was subtracted from all values. Methyltransferase extracts were obtained from *E. coli* RP4372 transformed with plasmid pGK3. Results are expressed as percentage of methyl esterification present in an equivalent amount of aspartate receptor in nonsolubilized membranes.

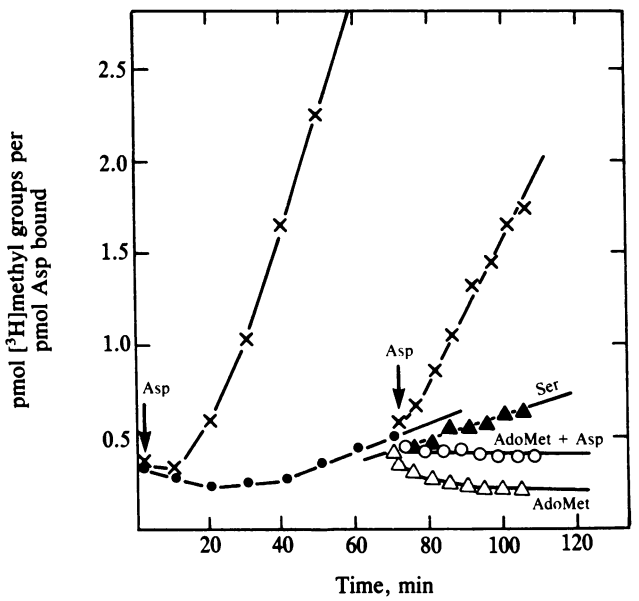

FIG. 3. Time course of methylation and demethylation of detergent-solubilized aspartate receptor in the absence and presence of aspartate at acidic pH. Aliquots of standard OcGlc extract/phospholipids/glycerol mixtures were incubated with methylation media containing 3 μM Ado[^{3}H]Met, RP4372(pDK1) cytosolic extract, and 25 mM Mes at pH 5.5, in the absence (●) and presence (×) of 1 mM aspartate. After 70 min at 32°C the sample that was not stimulated with aspartate was subjected to four alternative additions: (*i*) 1 mM serine (▲); (*ii*) 1 mM aspartate (×); (*iii*) 150 μM unlabeled AdoMet (△), or (*iv*) 150 μM unlabeled AdoMet plus 1 mM aspartate (○). At time points indicated, 10-μl samples were removed, and the radioactivity incorporated into carboxymethyl groups of proteins was determined as described for Fig. 2.

rate of the solubilized receptor shows stimulation by aspartate as observed *in vivo*.

In Fig. 5 is shown the effect of ionic strength on the stimulation factor. It is seen that increasing ionic strength diminishes the stimulation factor and in fact the ratio approaches 1.0 (no stimulation) at ionic strength 95 mM (20 mM from methylation medium + 75 mM from NaCl) at pH 6.5.

If the solubilized receptor is in essentially the same conformation as the receptor *in vivo*, its rate of methylation with comparable concentrations of transferase should be similar. The initial rate of methylation at pH 6.5 in 50 mM Mes at 100 μM AdoMet was 0.21 pmol·min^{-1} per pmol of aspartate bound. The concentration of methyltransferase and receptor obtained from the values of aspartate binding and 500-fold purification of methyltransferase (A. Russo, personal communication), 0.13 μM and 0.33 μM, respectively, allow us to calculate the turnover rate for the methyltransferase in the soluble system as 0.54 min^{-1}. *In vivo*, where the concentrations of methyltransferase and receptor are 1.4 μM and 32 μM, the turnover rate with overproduced receptor is 6 min^{-1}. (At sufficiently low transferase concentrations, the transferase *turnover rate* should be *independent* of transferase concentration.) Thus the substrate (receptor) concentration is lower in the solubilized system by a factor of 100, which more than adequately explains the decrease by a factor of 10 in the turnover rate.

Effect of Other Phospholipids. The effect of other phospholipids was explored and the results are shown in Table 1. Phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine gave essentially the same basal rate of methylation as the *E. coli* phospholipids mixture did. However, the extent of stimulation by aspartate was different in each lipid system. Phosphatidylethanolamine and phosphatidylcholine, which have in common a zwitterionic polar head, gave 1.0- and 1.3-fold stimulation, respectively. On the

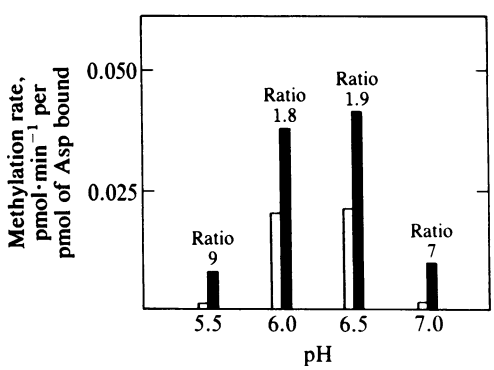

FIG. 4. Effect of pH on basal and aspartate-stimulated rates of methylation at a fixed ionic strength. Using cytosolic extracts from RP1091(pGK3) as the source of methyltransferase, we measured methylation of miscibility mixtures in 27 mM Mes buffer at pH values 5.5, 6.0, 6.5, and 7.0 and 3 μM Ado[^{3}H]Met in the absence (open bars) and presence (closed bars) of 1 mM aspartate. To compensate for the increase in ionic strength on raising the media pH, NaCl was added at concentration sufficient to bring the total ionic strength to 25 mM. Reactions were initiated by adding methylation media to the miscibility mixture, and 10-μl samples were removed at 6-min intervals over 60 min. Radioactivity incorporated into carboxymethyl groups of proteins was determined as described for Fig. 2, and initial rates of methylation were calculated from slopes of the curves obtained. Data are expressed as pmol of [^{3}H]methyl groups incorporated per min per pmol of aspartate bound to OcGlc extracts. Ratios indicate the ratio of initial rates of methylation upon aspartate stimulation to the basal rate.

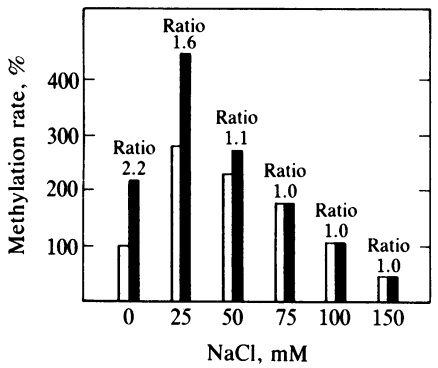

FIG. 5. Effect of salt concentration on basal and aspartate-stimulated rates of methylation at constant pH. Aliquots of standard OcGlc extracts/phospholipids/glycerol mixtures were incubated with cytosolic extracts from *E. coli* RP1091(pGK3) as described for Fig. 2. Each experimental mixture contained 27 mM Mes, pH 6.5, and increasing concentrations of NaCl, in the absence (open bars) and presence (closed bars) of 1 mM aspartate. Initial rates of methylation were determined as described in the legend to Fig. 4 and are expressed as percentage of basal rate of methylation at 0 mM added NaCl (20 mM ionic strength).

other hand, phosphatidylserine, phosphatidylglycerol, and cardiolipin did not allow methylation either in the absence or in the presence of aspartate. When phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin were added to the reconstitution media at a ratio similar to that present in the *E. coli* phospholipids mixture, both basal and stimulated rates of methylation increased.

Identification of the Sites of Methyl Esterification on the Solubilized Aspartate Receptor. To determine whether the methylation of detergent-soluble aspartate receptor occurs at the same sites previously identified (15), we analyzed radioactive peptides. Receptors in the solubilization mixture were methylated with cytosolic extracts from RP1091(pGK3) in 27 mM Mes, pH 6.0, in the absence and presence of aspartate. After sequential digestions with the proteases, the peptides were separated by reversed-phase HPLC. The results are shown in Table 2. The solubilized receptor was methylated at the same groups (Glu-294, Glu-302, Glu-309, and Glu-491) as

Table 1. Methylation of the aspartate receptor reconstituted with different phospholipids

Phospholipid	Methylation rate		Ratio, presence to absence of Asp
	Asp absent	Asp present	
E. coli mixture	100	186	1.8
Phosphatidylethanolamine (PtdEtn)	63	63	1.0
Phosphatidylcholine (PtdCho)	51	68	1.3
Phosphatidylserine (PtdSer)	0	0	—
Phosphatidylglycerol (PtdGro)	0	0	—
Cardiolipin (C)	0	0	—
PtdEtn/PtdGro (7:2, wt/wt)	145	242	1.6
PtdEtn/PtdGro/C (7:2:1, wt/wt)	145	339	2.3

OcGlc extracts were incubated with glycerol and the different preparations of phospholipids (1.8 mg/ml). Values of initial rates of methylation at pH 6.5 in 27 mM Mes buffer are expressed as percentage of the basal rate (aspartate absent) obtained with the *E. coli* phospholipids mixture. Conditions as for Fig. 5.

Table 2. Differential incorporation of methyl groups into the aspartate receptor methylated *in vitro* in the absence and presence of aspartate

Site	[^{3}H]Methyl incorporation, %		
	Soluble receptor		
	Asp absent	Asp present	Membranes
2	67.8	38.5	38.9
3	21.6	38.2	34.0
1	5.5	16.0	15.3
4	5.1	7.3	11.8

Values of radioactivity present in the four methylation sites were pooled for each sample and the relative amounts of radioactivity in individual sites were determined. Data are expressed as percentage of total radioactivity. Membranes were methylated by the standard procedure with added transferase as described for the solubilized receptor.

the membrane-bound receptor. Interestingly, the receptor esterified *in vitro* in lipid vesicles with no aspartate had the same distribution of methyl groups as the solubilized receptor in the presence of aspartate. Thus the receptor in the solubilization mixture appears to be very similar to the *in vivo* receptor as shown by sites of methylation, whereas the receptor reconstituted in vesicles appears to be in the aspartate-stimulated form even in the absence of aspartate.

DISCUSSION

The vectorial transmembrane receptor for aspartate sensing has been solubilized in functional form. Evidence that the detergent-treated receptor is in essentially the same conformation as the receptor *in vivo* is obtained from studies on aspartate binding, rates of methylation, sites of methylation, and stimulation of methylation rate by aspartate. In each case the solubilized receptor has essentially the same characteristics as the receptor *in vivo*. While complete identity could not be claimed, enzymatic rates and binding studies are highly sensitive quantitative evidence suggesting that the conformations are identical as far as many functional properties are concerned.

The value of such a solubilized receptor for understanding the chemotaxis system and the implications for receptor systems in general is illustrated in Fig. 1. A purified system is advantageous because defined components can be added back to it to explore kinetic and other properties. A reconstituted receptor in a lipid vesicle is valuable also, but it has experimental constraints as discussed in the introduction. A functional solubilized receptor can be studied in homogeneous solution by classical kinetic techniques. The experiments are simpler to perform, relatively free of ambiguities, and can be designed to measure such classic constants as K_m and V_{max}, which are then useful to explain function *in vivo*. In the present case the methylation kinetics are directly related to the adaptation properties of the bacterial sensing, and these kinetics can now be studied accurately and in detail.

The results described here support the general concepts relating detergents and proteins developed by Helenius, Simmons, Tanford, Clarke, *et al.* Since the aspartate-binding domain of this receptor appears to have the amino acid composition of a soluble protein, the detergents should not bind to this part of the protein and hence should not affect aspartate binding, as is observed. A globular piece appears to be on the cytoplasmic side of the membrane. This domain and the methyltransferase enzyme, which is soluble, should not be affected by detergent. Hence methylation kinetics should likewise be unaffected, provided a conformationally correct molecule has been reconstituted. Fig. 1*D*, showing detergent

around the membrane-transversing region, therefore fits the properties of the aspartate receptor and is in agreement with the theory of detergent interaction with proteins. That this membrane-spanning region is responsible for the integration of the receptor in the membrane is indicated by the fact that receptor, even when overproduced 20-fold, remains an integral membrane protein.

The method described here for obtaining a "solubilization mixture" that produces a functional receptor can probably be generalized, although the details of the mixture of phospholipids and glycerol may vary. Obviously the geometry and forces in the membrane-spanning region must be similar to those of the membrane *in vivo*. To achieve that, experimental testing of various combinations of phospholipid, detergent, solvent hydrophobicity, ionic strength, pH, etc., is necessary. In this case phospholipids, glycerol, and detergent at an appropriate pH and ionic strength provided the proper mixture. The phospholipids of the natural *E. coli* membrane were most effective, but they alone did not produce a functional receptor without added glycerol. Probably the same mixture will be useful for other receptors, but it would be expected that some changes in the composition of the solubilization cocktail will be required from case to case.

A solubilized receptor system cannot reveal all aspects of all transmembrane molecules. Thus, the vectorial release of ions must be studied in a membrane, but the solubilized receptor can still be used to elucidate properties such as a GTP-binding protein–adenylate cyclase reaction inside a membrane generated by an external stimulus. It can reveal the properties of covalent modification—e.g., autophosphorylation—now shown to be a characteristic of most receptors. It has the great advantage that accurate K_m and V_{max} values, inhibitor constants, etc., can be measured. The sensitivity of such assays, moreover, can detect nuances not discernible in more crude complexes *in vivo*. A soluble functional receptor adds a valuable experimental approach to the study of receptor function.

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