Proc. Natl. Acad. Sci. USA
Vol. 74, No. 5, pp. 1932–1936, May 1977
Biochemistry

Use of a distant reporter group as evidence for a conformational change in a sensory receptor

(galactose receptor/5-iodoacetamidofluorescein/chemotaxis/spectroscopic rules)

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Contributed by Daniel E. Koshland, Jr., February 28, 1977

ABSTRACT **A highly sensitive method for demonstrating ligand-induced conformational changes in protein molecules in solution is described. The method utilizes an environmentally sensitive reporter group that is known to be distant from the active site. In the present application a conformational change is demonstrated in the galactose receptor of *Salmonella typhimurium*, involved in bacterial sensing and transport, by means of an extrinsic fluorophore, 5-iodoacetamidofluorescein, attached at a single methionine residue, and the intrinsic tryptophan fluorophore. Binding of the ligand galactose perturbs the microenvironment of both the fluorescein and tryptophan, as shown by both spectral and potassium iodide quenching changes. The distance between the two dyes is established by fluorescence energy transfer methods to be 41 ± 10 Å. Since only one molecule of galactose binds per molecule of receptor and since the galactose molecule is only about 5 Å in length, changes at one of these sites reflect the result of an indirect effect. Hence, there must be a ligand-induced conformational change that is propagated a minimum of 30 Å through the receptor molecule.**

The ability to undergo ligand-induced conformational changes is now an accepted feature of protein molecules. To date, the most widely used method providing unequivocal and detailed evidence of this phenomenon has been x-ray crystallography. Crystallographic studies of hemoglobin (1), carboxypeptidase (2), and lactate dehydrogenase (3) have demonstrated that specific atoms in these protein molecules assume different spatial arrangements in the presence and absence of substrate. Protein crystallography has its limitations, however, which include its inapplicability to the dynamics of molecular interactions in solution, the difficulties of obtaining pure crystals, and the laborious nature of the procedure. Moreover, many protein molecules, of which receptors are a good example, are not available in the quantities required for such an approach.

Other methods that can complement or precede x-ray analysis are needed, therefore, for the study of conformational changes in proteins. One method that has provided definitive but not widely applicable evidence is demonstration of increased reactivity of specific protein residues upon ligand binding (4). Spectroscopic approaches, which include absorption spectroscopy, far-ultraviolet circular dichroism, and fluorescence, are simple to apply and frequently detect changes in the microenvironment of specific protein residues. Such methods as are conventionally used, however, do not distinguish between a direct effect, i.e., a change in the immediate vicinity of the bound ligand, and an indirect effect, i.e., a change in the microenvironment of a residue at some distance from the ligand-binding site.

A method is developed that is applicable to dynamic interactions and that also gives unambiguous evidence of conformational changes. The method utilizes a reporter group (5) known to be far from the active site as a probe of conformational changes that occur upon ligand binding. In the present application, two reporter groups are placed sufficiently distant from one another that the bound ligand cannot interact directly with both groups simultaneously. The distance between the dyes is established by fluorescence energy transfer measurements following Förster's equation (6) and Wu and Stryer's use of fluorescence as a spectroscopic ruler (7). A change in the microenvironment of the fluorescent groups then provides evidence for a delocalized conformational change and a description of the dynamics of the event.

The molecule chosen for development of this method was the galactose receptor of *Salmonella typhimurium* in bacterial sensing. The galactose binding protein of *Escherichia coli* was first isolated by Anraku (8, 9), and was shown to be the receptor in chemotaxis by Hazelbauer and Adler (10). Recently, Strange and Koshland (11) have described a model for receptor action that postulates a ligand-induced conformational change in the galactose receptor as an obligatory first step prior to its interaction with the signalling apparatus of the sensory system. Boos and coworkers (12–15) reported spectral changes associated with one or more tryptophan residues in the antigenically related galactose receptor of *E. coli* upon binding of galactose. These findings, together with the observation that the *S. typhimurium* protein contains a single tryptophan and binds a single galactose molecule (16), led us to utilize as the pair of reporter groups 5-iodoacetamidofluorescein (5-IAF) specifically attached at a single site on the protein and the single intrinsic tryptophan moiety of the *Salmonella* protein. The results and general applicability of the method are discussed.

MATERIALS AND METHODS

The galactose binding protein was isolated by described methods from *S. typhimurium* strain ST1 (16). The protein was determined to be homogeneous by gel electrophoresis under a variety of conditions. Protein concentrations were determined by the method of Lowry *et al.* (17) and binding activities by the nitrocellulose filter assay (18). Liquid scintillation counting was carried out in an Aquasol/toluene 2:1 mixture on a Packard Tricarb 3320 Liquid Scintillation Spectrometer.

The galactose-binding protein was modified by treatment with a 1000-fold excess of 5-iodoacetamidofluorescein (a gift of Richard Haugland, Hamline University) in 10 mM sodium phosphate buffer, pH 7.0/150 mM sodium chloride for 24 hr in the dark. The labeled protein was then purified by passage through a Sephadex G-25 column equilibrated with the same

Abbreviations: 5-IAF, 5-iodoacetamidofluorescein; 5-AF, 5-acetamidofluorescein.

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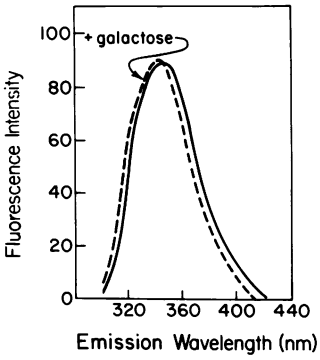

FIG. 1. Fluorescence emission spectra (uncorrected) of the *Salmonella* galactose receptor in the presence and absence of 10^{-4} M galactose. Spectra were recorded on a Perkin Elmer-Hitachi MPF-2A fluorescence spectrometer at ambient temperature with an excitation wavelength of 288 nm. Galactose binding protein (20 μg/ml) was in 10 mM sodium phosphate buffer, pH 7.0/150 mM NaCl.

buffer, and was subsequently dialyzed against this buffer for 5 days at 4° to remove carbohydrate contamination caused by passage through the Sephadex column.

The labeling ratio was determined from the protein concentration and the absorbance of the modified protein at 490 nm. A molar extinction coefficient of 42,000 for the attached fluorescein label at 490 nm was used. This value was obtained by comparison of the absorbance at 490 nm of the urea-denatured 5-acetamidofluorescein(AF)galactose receptor to the absorbance of the native AF-galactose receptor in buffer. A correction was made for the absorbance change of 5-IAF upon addition of urea. A correction for light scattering was made as described by Hartig *et al.* (19).

Steady-state fluorescence measurements were performed on a Perkin-Elmer Hitachi model MPF-2A fluorescence spectrometer at ambient temperature. Polarization measurements were made using the standard polarization accessory for the spectrophotofluorimeter, and were normalized for a constant grating transmission. The extent of energy transfer was calculated from the increase in the fluorescence excitation spectrum of the acceptor 5-AF at 280 nm.

The amino-acid compositions of the native and 5-AF-galactose receptors and their methionine sulfone derivatives were determined by Judy Benson and Marian Koshland using a Beckman model 120C amino acid analyzer. The methionine sulfone derivatives were prepared using performic acid reagent according to the method of Hirs (20).

RESULTS

Effect of Galactose on Intrinsic Fluorescence of *S. typhimurium* Galactose Receptor. In Fig. 1 is shown the fluorescence emission spectrum of the purified galactose binding protein in the presence and absence of saturating galactose. Galactose causes a 5 nm blue-shift in the tryptophan fluorescence spectrum. In contrast, no such shift is observed in the control on addition of sucrose, a sugar that shows no affinity for the protein (11). The spectrum of the tryptophan in the native protein has a maximum emission wavelength that is red-shifted relative to typical protein tryptophan emission, a finding which suggests a hydrophilic environment for this residue (21). The blue-shift that is observed upon addition of ligand usually indicates a change to a more hydrophobic microenvironment (21). A similar shift was observed by Boos in the *E. coli* protein (12).

Labeling of Receptor with a Fluorescent Reporter Group. The native galactose receptor can be reacted with 5-IAF to produce a covalently modified protein (5-AF-galactose binding protein), as shown by comigration of fluorescence at 520 nm with the single protein band during sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Modification proceeded to a fixed value, as shown in Fig. 2. At saturation, measurement of the absorbance at 490 nm of the protein in 8 M urea showed one fluorescent group per molecule of protein, as calculated from the molar extinction coefficient of the free dye in urea. Amino-acid analyses of the protein before and after modification established that a single methionine residue, and no other residues, was modified by this reagent (Table 1). Modification of one methionine was shown both by comparison of the methionine contents of galactose binding protein and 5-AF-galactose binding protein and more rigorously by comparison of the methionine sulfone content of the performate-treated proteins. The recoveries of lysine, histidine, and all other amino acids were found to be quantitative, in agreement with the absence of peaks in the positions at elution volumes identified with ε-carboxymethyllysine or 1-(or 3)-carboxymethylhistidine (22). Finally, a tryptic digest of the modified protein followed by paper electrophoresis in one dimension and chromatography in the second dimension showed modification of a single peptide. The reporter-modified galactose receptor retained full sugar-binding activity.

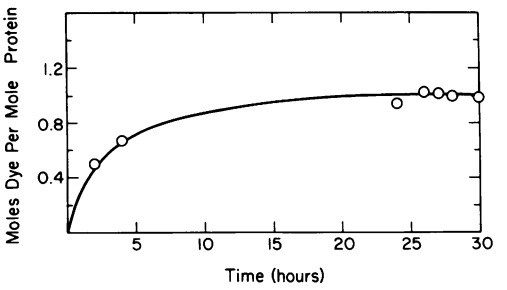

FIG. 2. Kinetics of labeling of the purified *Salmonella* galactose receptor. Galactose binding protein (0.5 mg/ml) was reacted with 5-IAF in 10 mM sodium phosphate buffer/150 mM NaCl at 22° in the dark. At specified times, an aliquot of the reaction mixture was removed and protein was separated from unreacted dye by passage over a Sephadex G-25 column equilibrated with the same buffer. The fraction containing galactose binding protein was assayed for protein by the Lowry *et al.* method (17) and for bound dye by absorbance at 490 nm using the appropriate corrections as described in *Materials and Methods.*

Effect of Galactose on Fluorescein Fluorescence of the 5-AF-Galactose Receptor. The fluorescence emission spectrum of the reporter group on the protein showed a 4 nm blue-shift and a 10% quenching upon binding of galactose at saturating

Table 1. Amino-acid analyses of the galactose receptor of *S. typhimurium* and its 5-AF-labeled analog

Amino acid	Galactose binding protein	5-AF-galactose binding protein
Lysine	31.7	31.1
Histidine	3.1	2.9
Aspartate	50.0	49.4
Cysteine	0	0
Methionine	5.7	4.8
Carboxymethyllysine	0	0
Carboxymethylhistidine	0	0
Methionine sulfone*	5.7*	4.6*

* Value reported is for the performate-treated protein.

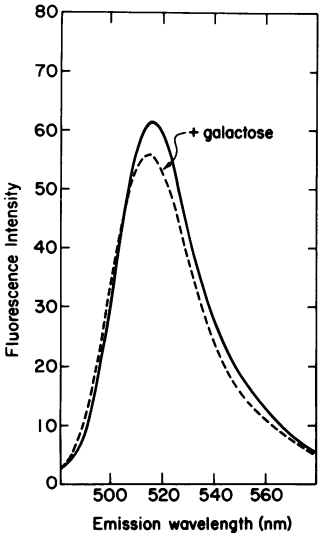

FIG. 3. Fluorescence emission spectra (uncorrected) of the 5-AF-modified galactose receptor from *Salmonella* in the presence and absence of 10^{-4} M galactose. Spectra were recorded at ambient temperature with an excitation wavelength of 460 nm. The 5-AF-galactose protein (10 μg/ml) was in 10 mM sodium phosphate buffer, pH 7.0/150 mM NaCl.

levels (Fig. 3). Changes of this type most likely indicate that the fluorescein residue is shifted to a more hydrophilic environment (19, 23).

KI Quenching. Potassium iodide (KI) quenching (24) of fluorescence emission in the galactose binding protein in the presence and absence of substrate was examined (Fig. 4). In the absence of substrate, KI quenching of the protein fluorescence is observed; a linear response of $F_0/\Delta F$ against 1/[KI] with increasing KI is observed. The presence of 10^{-4} M galactose decreases the rate of quenching by KI, a result which shows that ligand reduces access to the tryptophan residue.

In the case of the modified receptor, a linear quenching of the fluorescein fluorescence is observed and differs little from the quenching behavior of 5-IAF in solution. Addition of saturating amounts of the sugar galactose causes a small increase in the quenching rate (Fig. 4 lower). These findings suggest a very accessible 5-IAF binding site, which becomes even more accessible upon ligand binding.

Distance between the Two Reporter Groups. The distance between two fluorophores that undergo excitation transfer can be calculated from the theory of Förster (6), as demonstrated experimentally by Wu and Stryer (7). The greatest ambiguity in application of this theory is determination of the orientation factor, K^2, which appears in the Förster equation (Eq. 1).‡

$$\frac{9(\ln 10)\phi_D K^2}{128\pi^5 N' n^4 \tau_D R^6} J(\tilde{v}) = K_t \qquad [1]$$

‡ Symbols are as follows: Φ_D is the quantum yield of the donor in absence of acceptor; N' is Avagadro's number; n is the refractive index of the intervening medium; R is the donor-acceptor separation; $J(\tilde{v})$ is the overlap integral of the normalized donor emission spectrum with the absorption spectrum; P_{om} is the polarization of a fluorophore bound to a macromolecule when the macromolecule is immobilized, but the fluorophore is free to move within the restricted environment of its binding site; P is the observed polarization; τ is the fluorescent lifetime; ρ_0 is the rotational correlation time of a sphere of equivalent volume to the macromolecule; $N_1 = \rho_1/\rho_0$ and $N_2 = \rho_2/\rho_0$ where ρ_1 is the rotational correlation time for the long axis of the ellipse and ρ_2 for each of the two short axes; P_0 is the immobilized polarization for the fluorophore; and K^2 is dipole-dipole orientation factor (6,7).

Table 2. Polarization of fluorescence values for 5-IAF free and on the modified galactose binding protein*

Theoretical or observed system	Polarization value
5-IAF in H_2O	0.017†
P_0 for 5-IAF	0.43
Theoretical maximum for 5-AF randomly immobilized on protein of molecular weight 33,000 and axial ratio: 1:1	0.32
2:1	0.325
4:1	0.36
5-AF-GBP‡	0.11
5-AF-GBP in 5×10^{-4} M galactose	0.12
5-AF-GBP in 10 M urea	0.06
5-AF-GBP in 10 M urea and 5×10^{-4} M galactose	0.06

* Excitation wavelength, 490 nm; emission wavelength, 520 nm.
† From ref. 19.
‡ 5-AF-galactose binding protein.

K^2 can assume any value between 0 and 4, but it is frequently assumed to be 2/3, the value for a donor and acceptor pair that is free to sample all angular orientations by rapid tumbling. The latter assumption cannot be made in the case of fluorescent groups covalently attached to proteins. For the case of fluorophores restricted in their motion, Dale and Eisinger (25, 26) have developed a modeling approach in which each fluorophore is assumed either to be immobilized or to execute rapid motion throughout the defined volume or surface area of a cone. The cone half-angle is calculated from polarization measurements for the dye, and the orientation factor, K^2, is estimated from this computed value.

Our theoretical approach follows that of Dale and Eisinger except that (*i*) from the equations of Perrin (27) and Weber (28), P_{om} has been calculated from the known macromolecular shape and steady-state polarization measurements, and (*ii*) the Weber equation for a dye attached with random orientation to a prolate ellipsoid has been substituted for that used by Dale and Eisinger for a spherical macromolecule. The resulting equation that relates P_{om} to P is given in Eq. 2:¶

$$\left(\frac{1}{P}-\frac{1}{3}\right) = \frac{\left(\frac{1}{P_{om}}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\rho_0 N_1}\right)\left(1+\left[\frac{4}{N_2}-\frac{1}{N_1}\right]\frac{\tau}{\rho_0}\right)}{1+\left(\frac{5}{N_2}+\frac{1}{N_1}\right)\frac{\tau}{2\rho_0}-\frac{9}{8}\left[\frac{\left(\frac{1}{N_2}-\frac{1}{N_1}\right)^2\left(\frac{\tau}{\rho_0}\right)^2}{\left(1+\left[\frac{2}{N_1}+\frac{1}{N_2}\right]\frac{\tau}{\rho_0}\right)}\right]} \qquad [2]$$

The measured polarization for 5-AF bound to the galactose receptor protein is 0.11 (Table 2). This value falls between the polarization observed for the free dye in water, 0.017 (19), and the theoretical value, 0.324, calculated for this dye randomly

¶ Deviations from the assumption of an isotropic distribution of 5-AF orientations relative to the ellipsoid axes are not critical in many cases. For example, in this case with a 2:1 axial ratio, the relaxation time calculations are very much like those for a sphere of equivalent volume. Application of a spherical model to 5-AF-labeled galactose receptor yields a conical motion half-angle of 47°, in close agreement with calculations presented herein. A more complete discussion of these models will be presented elsewhere.

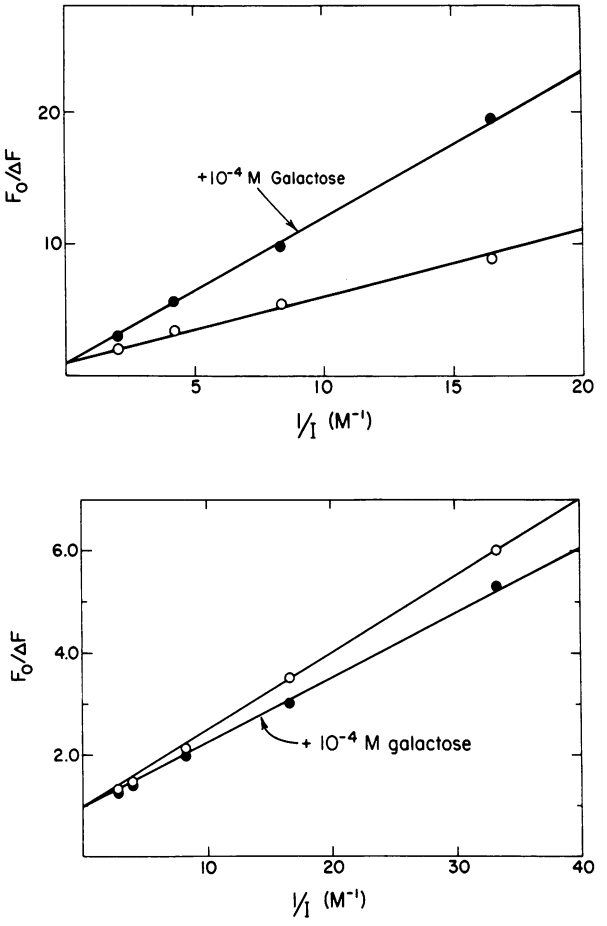

FIG. 4. Quenching of protein fluorescence emission by potassium iodide. To (*Upper*) galactose binding protein (20 μg/ml in 0.01 M sodium phosphate, pH 7.0/150 mM NaCl) or to (*Lower*) 5-AF-labeled galactose binding protein (10 μg/ml in the same buffer) in the presence (●) and absence (○) of 10^{-4} M galactose, increasing amounts of 6 M KI were added. A control cuvet received increasing amounts of 6 M NaCl. F_0, fluorescence in the NaCl-containing control cuvet; ΔF, difference in fluorescence of sample and control cuvet. $1/I^-$ was calculated using the proper dilution factor. (*Upper*) Excitation wavelength, 288 nm; emission wavelength, 340 nm. (*Lower*) Excitation wavelength, 460 nm; emission wavelength, 517 nm.

immobilized on a protein of 33,000 molecular weight with a 2:1 axial ratio for a prolate ellipsoid [the values for the galactose binding protein (29)]. Thus, the reporter group has considerable, but not complete, freedom of motion. These polarization values were then used in Eq. 2 to give a P_{om} of 0.151. The latter was applied according to the Dale and Eisinger theory (Fig. 52 of ref. 25) to give a cone half-angle of 48° for 5-AF. With this value for K^2 of the fluorescein reporter group and assuming that the tryptophan residue is immobilized, the limits of K^2 are calculated to be 0.15–2.82.

The energy transfer efficiency from the donor tryptophan to the acceptor, 5-AF, was determined to be 22% from the excitation spectrum of 5-AF bound to the protein. Using the Förster equation (Eq. 1), and assuming the usual values for other terms (7), an efficiency of 22% and a K^2 value of 2/3 give a distance of 41 Å. The calculated limits of K^2 from the above theory give a range of 32–52 Å for the distance between tryptophan and 5-AF. Since the galactose molecule is only 5 Å in length (Fig. 5) and only one is bound per molecule of protein (16), it is clear that galactose cannot interact directly with both groups.

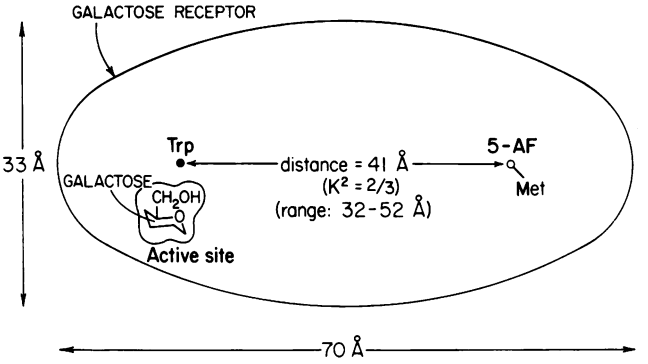

FIG. 5. Schematic representation of the *Salmonella* galactose receptor showing the dimensions of the protein, based on an axial ratio of 2:1, and the distance from the single tryptophan to the 5-AF labeling site as calculated from fluorescence energy transfer measurements. The model includes the single galactose binding site and nearby tryptophan by analogy to the antigenically related *E. coli* arabinose receptor (30).

DISCUSSION

By fluorescence energy transfer measurements we have determined that the single tryptophan residue and the single acetamidofluorescein moiety of the 5-IAF-modified *Salmonella* galactose receptor are 41 ± 10 Å apart. Since (*a*) the galactose molecule is only 5 Å in length, (*b*) only one molecule of ligand binds per molecule of receptor, and (*c*) its binding perturbs the microenvironments of both the tryptophan and fluorescein fluorophores, it is clear that the changes introduced at a minimum of one of these sites are the result of an indirect effect. Hence, there must be a ligand-induced conformational change that is propagated a minimum of 30 Å through the prolate receptor molecule.

This "distant reporter group method" requires the placing of an environmentally sensitive group at a position known to be too distant to allow direct interactions with the ligand. In this application one intrinsic fluorophore, the tryptophan residue, and one dye added to a particular amino acid residue were utilized. However, two added reporter groups or a fluorescent ligand plus a fluorescent reporter group could serve equally well. For example, many enzymatic reactions involve substrates or allosteric effectors that are fluorescent or that have analogs that compete for the binding site that are fluorescent. Establishing that the reporter group is distant from the binding site by energy transfer between the reversibly bound ligand and the reporter group would be sufficient. The donor-acceptor pair must be chosen to allow the fluorescence transfer measurements and the distant group must show microenvironment changes on ligand binding. If two reporter groups are introduced, it will usually be convenient to use different covalent linkages, e.g., one alkylating and one acylating, to attach the dyes to different amino acid residues. The use of fluorescent reporter groups was a particularly attractive feature of the method since the great sensitivity of fluorescence facilitates subsequent dynamic studies of such labeled receptors. However, any method that can establish distances unequivocally would satisfy the procedure.

This approach is less demanding than mapping positions on protein surfaces by fluorescent energy transfer measurements. In the present application only a minimum distance need be established. If that minimum (in this case, 30 Å) is far greater than the size of the ligand (in this case, 5 Å), the chance for an erroneous conclusion is very remote. As the size of the ligand increases and the distance between reporter groups decreases the chance for error will increase, but this problem can be

controlled by the investigator since there are many different reporter groups that can be attached to proteins.

The above derivation makes a number of assumptions (more detailed calculations and theory will be published elsewhere), but all are based on widely observed experimental results or affect the final numbers in minor ways. For example, it is assumed that the tryptophan is immobile, a fact supported by the confined space of the tryptophans so far studied in protein structures and their line widths in nuclear magnetic resonance studies, e.g., the histidine receptor study of Robertson *et al.* (31). The 5-AF is assumed to execute rapid motion in the cone of its oscillation, but deviations from isotropic motion do not invalidate the method since the calculation of the cone half-angle and the K^2 values are affected in the same way by deviations from isotropic conical motion. Similarly, the calculated distance is not very sensitive to the shape of the protein. Calculated ranges of K^2 vary from 0.16 to 2.73 for a 4:1 prolate ellipsoid and from 0.15 to 2.82 for a sphere. A mobile tryptophan would in fact diminish the range error.

Several pieces of data independently corroborate the finding of a ligand-induced conformational change in the labeled galactose receptor. First, modification of a methionine with the large, bulky fluorescein moiety results in no detectable loss of biological activity. If sugar binding involved direct interaction with this amino acid, a substantial loss of activity would probably be associated with its chemical modification. Second, the finding that the fluorescent group becomes more accessible to potassium iodide upon ligand binding demonstrates a conformational change, since a direct interaction would be expected to bury this residue. Third, the distance between the groups is consistent with the dimensions of the protein molecule. A prolate ellipsoidal protein of molecular weight 33,000 and axial ratio 2:1 has dimensions of 33 Å × 33 Å × 70 Å as a prolate ellipsoid. Dimensions of *68* Å × *38* Å × *30* Å are observed from crystallographic data of the similar arabinose binding protein (30, 32). Hence, a distance of 41 ± 10 Å for the two reporter groups is in agreement with the independent limitations of protein size. Finally, the previous work of Boos and coworkers, in which various spectroscopic tools were used to demonstrate conformational changes in the *E. coli* galactose receptor, was subject to the ambiguities described above, but, nevertheless, is entirely consistent with an induced conformational change. Such a conformational change is, moreover, supported by the finding that only one molecule of galactose binds per molecule of receptor (16).

It is generally assumed that effectors induce conformational changes in the receptors to which they bind. The evidence for such a mechanism is usually circumstantial, however, both because of the difficulty of isolating receptors and the ambiguity of the experimental tests. The finding that the purified *Salmonella* galactose receptor must undergo a conformational change on binding of its ligand is, therefore, although not surprising, important support for a widespread belief. It is of particular interest in this case because previous studies of this sensory system had led to the hypothesis that a conformational change must be induced in the free receptor prior to its association with the first component of the signaling system. The demonstration that the isolated purified protein does undergo such a conformational change, therefore, adds further support for such an association mechanism for receptor action.

We thank Dr. Terry Leighton for assistance and advice with peptide chromatography and Ms. Judy Benson and Dr. M. E. Koshland for assistance and advice in the amino-acid analyses. The work was funded by U.S. Public Health Service Grant AM9765 and National Science Foundation Grant PCM76-21850.

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