References

Antonini, E., and Brunori, M. (1971), *in* Hemoglobin and Myoglobin in Their Reactions with Ligands, Amsterdam, North Holland Publishing Co., pp 31–32.
Benesch, R. E., Benesch, R., and MacDuff, F. (1964), *Biochemistry 3*, 1132.
Benesch, R. E., Benesch, R., and Williamson, M. E. (1962), *Proc. Nat. Acad. Sci. U. S. 48*, 2071.
Benesch, R. E., Benesch, R., and Yu, C. I. (1969), *Biochemistry 8*, 2567.
Bookchin, R. M., and Nagel, R. L. (1971), *J. Mol. Biol. 60*, 263.
Briehl, R. W., and Hobbs, J. F. (1970), *J. Biol. Chem. 245*, 538.
Bunn, H. F. (1972), *in* Hemoglobin and Red Cell Structure and Function, Brewer, G., Ed., New York, N. Y., Plenum Press, p 41.
Bunn, H. F., Ransil, B. J., and Chao, A. (1971), *J. Biol. Chem. 246*, 5273.
Drabkin, D. L. (1946), *J. Biol. Chem. 164*, 703.
Drysdale, J. W., Righetti, P., and Bunn, H. F. (1971), *Biochim. Biophys. Acta 229*, 42.
Edelstein, S. J., Rehmar, M. J., Olson, J. S., and Gibson, Q. H. (1970), *J. Biol. Chem. 245*, 4372.
Guidotti, G. (1967a), *J. Biol. Chem. 242*, 3685.
Guidotti, G. (1967b), *J. Biol. Chem. 242*, 3694.
Guidotti, G., Konigsberg, W. H., and Craig, L. C. (1963), *Proc. Nat. Acad. Sci. U. S. 50*, 774.
Huisman, T. H. J., and Dozy, A. M. (1965), *J. Chromatogr. 19*, 160.
Kellett, G. L., and Schachman, H. K. (1971), *J. Mol. Biol. 59*, 387.
Macleod, R. W., and Hill, R. J. (1973), *J. Biol. Chem. 248*, 100.
Nagel, R. L., and Gibson, Q. H. (1971), *J. Biol. Chem. 246*, 69.
Nagel, R. L., and Gibson, Q. H. (1972), *Biochem. Biophys. Res. Commun. 48*, 959.
Olson, J. S., and Gibson, Q. H. (1972), *J. Biol. Chem. 247*, 3662.
Park, C. M. (1970), *J. Biol. Chem. 245*, 5390.
Park, C. M. (1973), *Ann. N. Y. Acad. Sci. 209*, 237.
Perutz, M. F. (1970), *Nature (London) 228*, 726.
Thomas, J. O., and Edelstein, S. J. (1972), *J. Biol. Chem. 247*, 7870.
Tomita, S., Enoki, Y., Ochiai, T., and Kawase, M. (1973), *J. Mol. Biol. 73*, 261.
van Assendelft, O. W. (1970), Spectrophotometry of Haemoglobin Derivatives, Springfield, Ill., C. C Thomas.
Vinograd, J., and Hutchinson, W. D. (1960), *Nature (London) 187*, 216.

Involvement of a Tryptophan Residue in the Binding Site of *Escherichia coli* Galactose-Binding Protein†

Eleanor B. McGowan,‡ Thomas J. Silhavy, and Winfried Boos*

ABSTRACT: The galactose-binding protein, a component of the β-methyl galactoside transport system of *Escherichia coli*, undergoes a conformational change upon the binding of substrate. A variety of spectrophotometric techniques were employed in order to probe the nature of this change. All known substrates of the β-methyl galactoside transport system cause an alteration in the ultraviolet absorbance of the galactose-binding protein. The ultraviolet difference spectra produced by these substrates are similar but not identical and resemble solvent perturbation difference spectra of *N*-acetyltryptophan ethyl ester. In addition, solvent perturbation difference spectroscopy reveals that the exposure of external chromophores is not affected by binding of substrate. These results indicate that the substrate interacts directly with a tryptophan residue present in the binding site of the protein, and further, that the active site tryptophan is not accessible to the bulk solvent either in the presence or absence of substrate. However, substrate protects the protein from fluorescence quenching by KI. It is concluded that the alteration in surface charge of the protein which has been shown to accompany the binding of substrate causes this differential quenching of an external tryptophan. A mutant galactose-binding protein which does not exhibit an increase in electrical charge upon substrate binding is not protected from the quenching effects of KI by substrate, even though interaction of substrate with the active-site tryptophan still occurs. Thus, the substrate-dependent conformational change of the galactose-binding protein does not result in extensive refolding of the polypeptide chain, but rather is restricted to small alterations in the active site and an increase in the surface charge of the protein.

The galactose-binding protein from *Escherichia coli* is a periplasmic protein (Anraku, 1968a–c) necessary for the proper functioning of the β-methyl galactoside transport system (Boos, 1969, 1972; Boos and Sarvas, 1970; Lengeler *et al.*, 1971). Although the role of this protein in the transport mechanism is not understood, a conformational change has

† From the Department of Biological Chemistry, Harvard Medical School, and the Biochemical Research Laboratory, Massachusetts General Hospital, Boston, Massachusetts 02114. *Received August 27, 1973.* Supported by grants from the National Institutes of Health (GM-18498 and AM-05507) and National Institutes of General Medical Sciences Training Grant GM-00451 (T. J. S.).

‡ Present address: Department of Biochemistry, State University of New York Downstate Medical Center, Brooklyn, N. Y. 11203.

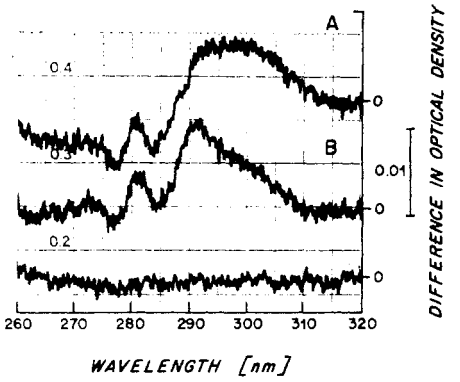

FIGURE 1: Ultraviolet difference spectra of galactose-binding protein caused by galactose and glucose. Protein solution (1 ml; 16 μM in 0.01 M Tris-HCl, pH 7.3) was placed in two matched quartz cuvets. A base line was obtained from 320 to 260 nm. Glucose (10 μl) (A) or galactose (10 μl) (B), final concentration 10 mM, was added to the sample cell and 10 μl of water was added to the reference cell, and the resultant spectrum was recorded.

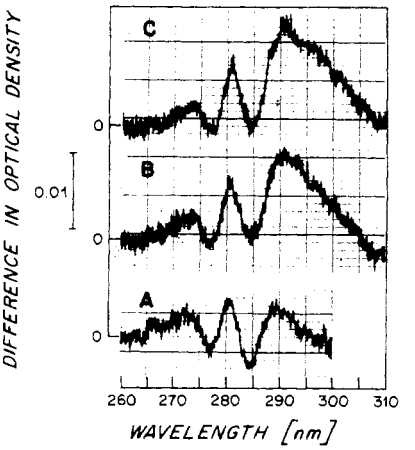

FIGURE 2: Dependence of the spectral change on the anomeric state of galactose. Protein (18 μM) was prepared as in Figure 1. Freshly prepared α-galactose was added to the sample cell at a final concentration of 80 μM. Spectra were obtained 2 min (A), 15 min (B), and 25 min (C) after dissolving the galactose.

been proposed to be the underlying mechanism of its function in transport (Boos and Gordon, 1971; Rotman and Ellis, 1972). Previous studies with the purified protein have shown that the binding of galactose is accompanied by a conformational change detected by an increase in electrophoretic mobility at pH 8.4 in the presence of galactose, altered binding constants for galactose, and a change in the fluorescence emission spectrum (Boos *et al.*, 1972).

Fluorescence studies have indicated that the environment of a tryptophan residue was altered by the binding of galactose. However, the nature of this alteration was unclear. The binding of galactose could result in a refolding of the peptide backbone and thus alter the environment of one or more tryptophan residues, or the galactose could interact directly with a tryptophan residue in the binding site, resulting in the observed change. The present study was undertaken to examine more closely the involvement of tryptophan residues in the binding process and resultant conformational change. Changes in the ultraviolet absorbance spectrum of the protein, accessibility of tryptophan and tyrosine residues to perturbation by bulk solvents, and the ability of galactose to protect the protein from the quenching of fluorescence emission by potassium iodide were examined. For comparison similar studies with a mutant galactose-binding protein were done. This protein exhibits a strongly reduced binding affinity, although the same specificity as the wild-type protein, and has lost the ability to increase its electrophoretic mobility upon binding substrate (Boos, 1972).

Materials and Methods

Galactose-Binding Protein. Wild-type galactose-binding protein was prepared from *E. coli* W3092cy$^-$, a galactokinase-negative strain. Cold osmotic shock, protamine sulfate treatment, and ammonium sulfate precipitation were carried out as described by Anraku (1968a). The protein obtained after the second ammonium sulfate precipitation was dialyzed against 0.01 M Tris-HCl (pH 7.3) and then against distilled water. Isoelectric focusing in a 440-ml LKB column was performed as described previously (Boos, 1972). The fractions showing antibody precipitin lines were pooled and dialyzed against 0.01 M Tris-HCl (pH 7.3) and concentrated to 5–10 ml using an Amicon ultrafiltration apparatus. The protein solution was applied to a 4 × 120 cm column of Bio-Gel P-150, 100–200 mesh (Bio-Rad), equilibrated with the same buffer. Absorbance at 280 nm and antibody reactivity were determined. The purified protein was pooled, precipitated by dialysis against saturated ammonium sulfate containing 0.1 M Tris-HCl (pH 7.3), and stored as a slurry at 4°. Before use, the protein was freed of ammonium sulfate by gel filtration on a 1.2 × 15 cm column of Sephadex G-100 equilibrated with 0.01 M Tris-HCl (pH 7.3). Protein concentration was determined by the biuret reaction (Gornall *et al.*, 1949). Purity was assessed by gel electrophoresis in sodium dodecyl sulfate as described by Boos and Gordon (1971). Mutant galactose-binding protein from *E. coli* EH3039 was prepared as described by Boos (1972).

Chemicals. Carbohydrates used were as previously described (Boos, 1972), and had the D configuration unless otherwise noted. Deuterium oxide (D_2O), 99.7%, was obtained from Sigma. All other chemicals were of reagent grade. Distilled deionized water was used throughout.

Ultraviolet Difference Spectroscopy. Ultraviolet difference spectra of protein in the presence and absence of various sugars were obtained using a Cary 15 double-beam recording spectrophotometer equipped with a 0–0.1 absorbance scale. The protein solutions were passed through a Millipore filter (0.45 μ) to remove any particles. Matched 1-cm, 1-ml quartz cells were used. Protein was placed in both cells, and a base line from 320 to 260 nm was obtained. A small aliquot (1–10 μl) of galactose or other sugar was added to the sample cell, and an equal amount of water was added to the reference cell. Galactose was dissolved in water at least 1 hr before use unless otherwise specified. The contents were mixed by inversion, and the resultant spectrum was recorded twice. The temperature was 23–24°.

Solvent Perturbation Difference Spectroscopy. Solvent perturbation difference spectra in the presence of 20% (v/v) methanol, ethylene glycol, dimethyl sulfoxide, or 90% (v/v) D_2O were obtained as described by Herskovits and Laskowski (1960, 1962), using cylindrical quartz tandem cells.

Fluorescence Spectroscopy. Florescence measurements were performed as previously described (Boos *et al.*, 1972). Potassium iodide quenching of fluorescence emission was performed as described by Lehrer (1967). The decrease in fluorescence emission of the protein in the presence or absence of galactose was followed as a function of potassium iodide concentration at 330 nm for the wild-type protein and at 336 nm for the

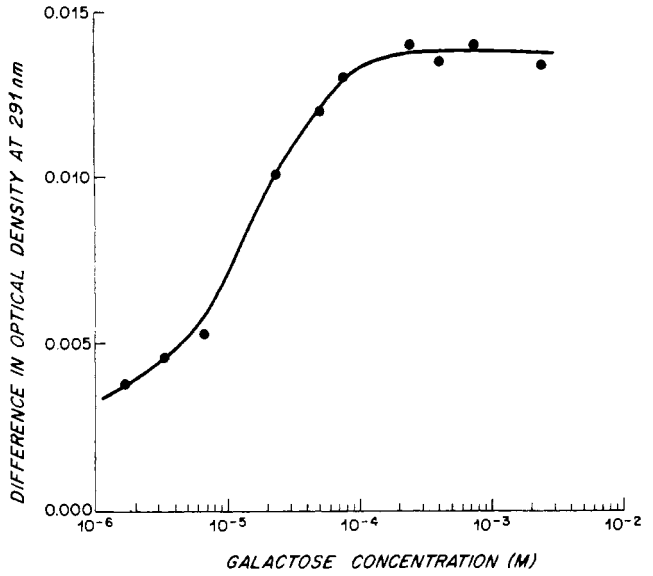

FIGURE 3: Concentration dependence of the galactose-induced spectral change. The protein solution was prepared as in Figure 1. Galactose was added in 1–5-μl aliquots of 10 mM to 1 M solutions to the sample cell and an equal volume of water was added to the reference cell. The increase in $\Delta A_{291\ nm}$ was determined.

mutant protein.

pH measurements were made using a Radiometer PHM 26 pH meter with a 10-fold scale expander.

Results

Ultraviolet Difference Spectra of the Galactose-Binding Protein Induced by Galactose and Glucose. Addition of galactose or glucose to the galactose-binding protein results in altered ultraviolet absorbance. The changes in the presence and absence of sugar were determined as difference spectra with the maximum difference (Figure 1), representing about 3% of the total absorbance of the samples. The difference spectra caused by galactose and glucose are similar but not identical, and resemble solvent perturbation difference spectra of *N*-acetyltryptophan ethyl ester (Herskovits and Sorensen, 1968), with maxima at 274, 281, and 291 nm. The $\Delta E_{291\ nm}$ values for galactose and glucose are 1200 and 1000 M^{-1} cm^{-1}, respectively. Both spectra have positive absorbance above 295 nm, higher than that observed with model tryptophan compounds, but similar to that observed with lysozyme in the presence of its substrate analog glycol chitin. In this case, the difference spectrum was shown to result from the interaction of the substrate analog with a specific tryptophan residue, removing it from contact with the aqueous environment (Hayashi *et al.*, 1963). The similarity but nonidentity of the two spectra suggests that the absorbance of a tryptophan residue in the active site is being affected somewhat differently by the two sugars. Other tryptophan residues may also contribute to the absorbance changes because of a conformational change occurring upon binding of the sugar.

Addition of freshly prepared α-galactose to a solution of the protein resulted in a spectrum (Figure 2A) markedly different from that obtained when equilibrated (70% β) galactose was added (Figure 1B). Spectrum A, completed 2 min after addition of water to crystalline α-galactose, shows a much smaller maximum at 291 nm than that obtained with galactose mutarotated to its equilibrium mixture. The time dependent increase in the 291-nm maximum is seen by comparing the spectra obtained at 2, 15, and 25 min. Spectrum C is the same as that obtained when equilibrated galactose is

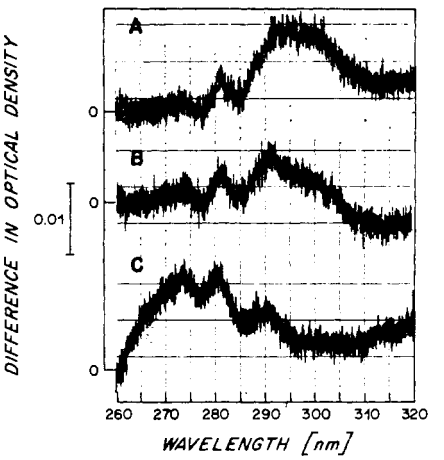

FIGURE 4: Effect of pH on the spectral change induced by galactose. Protein (16 μM) was dissolved in 0.1 M buffers of pH values 4.3–10.3. Galactose at 0.1 mM final concentration was added to the sample cell and water to the reference cell. The difference spectrum at pH 7.3 (A) is representative of spectra between pH 5.3 and 9.3; B, spectrum obtained at pH 4.3; C, spectrum obtained at pH 10.3.

added. The other two maxima do not increase in magnitude with time. This result indicates that both forms of galactose are bound by the protein, and that the configuration of the C-1 hydroxyl group affects the absorbance of a tryptophan residue in the active site.

Addition of increasing amounts of galactose to a solution of protein resulted in increased magnitude of the difference spectrum until a plateau value was reached (Figure 3). All peaks increased in magnitude with increasing sugar concentration; the largest peak, at 291 nm, was followed for convenience. At a protein concentration of 18 μM, no further increase in the 291-nm peak was observed above 100 μM galactose. The half-maximal change occurs at a calculated *free* galactose concentration of 1 μM. Similar results were obtained with glucose.[1] The results indicate that a specific, saturable response is obtained, rather than an effect on the protein of changing the bulk solvent composition.

Difference spectra induced by galactose were obtained with the protein in both cells at pH values ranging from 4.3 to 10.3. The spectra obtained at pH 5.3, 6.3, 7.3, 8.3, and 9.3 were virtually identical (Figure 4). At pH 4.3, the spectrum was similar but smaller in magnitude, while at pH 10.3 the spectrum was markedly different, with a large decrease in the 291-nm peak and an increase but no wavelength shift in the other two peaks. The marked difference in the spectrum at pH 10.3 may be due in part to a small difference in tyrosyl ionization.

Specificity of the Sugar-Induced Difference Spectrum. Sugars other than galactose and glucose were added to the galactose-binding protein to determine whether they could induce a spectral change. L-Arabinose, D-fucose, and (*R*)-glyceryl β-D-galactopyranoside gave rise to spectra similar to that observed with galactose. L-Glucose, D-mannose, D-allose, D-gulose, 2-glyceryl β-D-galactopyranoside, and lactitol did not give rise to any spectral change. The sugars which give rise to absorbance changes also increase the fluorescence emission spectrum of the protein (Boos *et al.*, 1972; Parnes and Boos, 1973) and are transported by the β-methyl galactoside transport system (Parnes and Boos, 1973).

[1] Free substrate concentration was calculated assuming that only one of the two binding sites was occupied at substrate concentrations below 1 μM (Boos *et al.*, 1972).

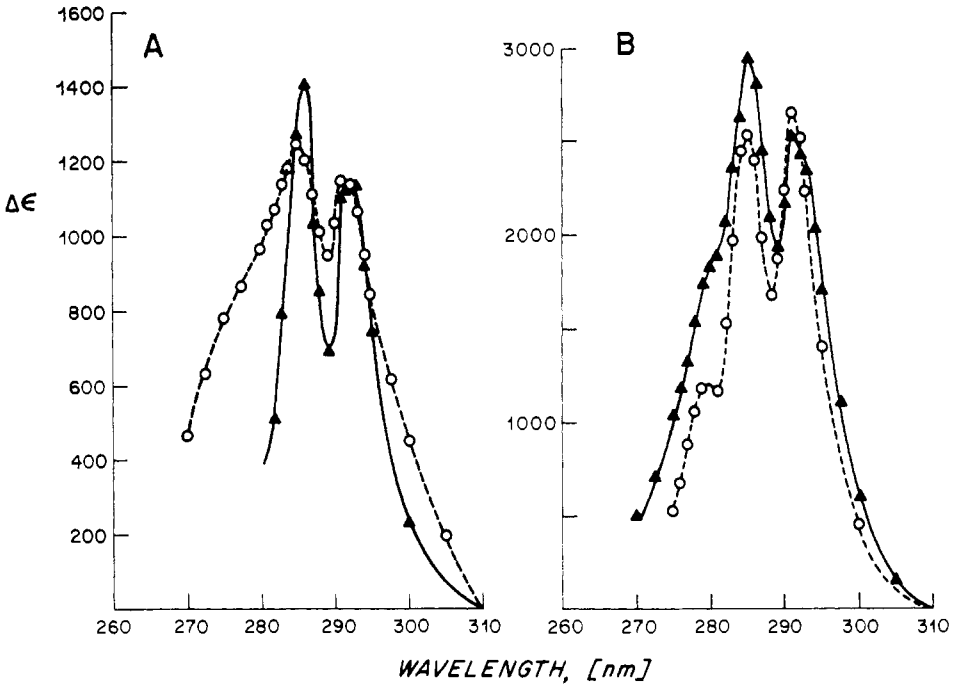

FIGURE 5: Solvent perturbation difference spectra of the galactose-binding protein. (A) Protein (20 μM in 0.01 M Tris-HCl, pH 7.3) was prepared in the presence and absence of 20% (v/v) Me_2SO as described by Herskovits and Laskowski (1960, 1962). (▲) Best fit (5 tyrosine, 2 tryptophan residues) calculated from the model compound data of Herskovits and Sorensen (1968); (○) observed spectrum. (B) Galactose-binding protein was prepared in 10 M urea, 0.01 M Tris-HCl, (pH 7.3). A final concentration of 8 M urea was obtained by adding Me_2SO or water to the sample in a 1:4 ratio. The protein concentration was 15 μM. (▲) Best fit (7 tyrosine, 5 tryptophan) calculated from the model compound data of Herskovits and Sorensen (1968). (○) Observed spectrum.

Solvent Perturbation Difference Spectra. This technique was utilized to determine the relative exposure of the chromophores in the protein and also to determine if this exposure is altered as a consequence of the substrate-induced conformational change. The galactose-binding protein, denatured by 8 M urea, contains 7 tyrosine and 5 tryptophan residues which are accessible to all perturbants used. Figure 5B shows the spectrum obtained with 20% Me_2SO and the calculated curve for 7 tyrosine and 5 tryptophan residues using model compound values (Herskovits and Sorensen, 1968). Amino acid analyses by Anraku (1968b) and spectroscopic determination of tyrosine and tryptophan by the method of Edelhoch (1967) gave the same composition.[2] In the absence of urea 4–5 of the tyrosine and 2 of the tryptophan residues are perturbed by (20% v/v) methanol, ethylene glycol, and Me_2SO. The smallest perturbant available, deuterium oxide (D_2O) (90% v/v), perturbed two additional tryptophan residues. Figure 5A shows the spectrum obtained with Me_2SO in comparison to the calculated curve for 5 tyrosine and 2 tryptophan residues.

The solvent perturbation difference spectra are further enhanced by the addition of substrate to the protein in the sample cell alone. Figure 6 shows the spectra with Me_2SO (A) and D_2O (B) as perturbants. Manual subtraction of the two curves shown in Figure 6A or B indicated that the observed alteration was identical with the galactose-induced difference spectrum of the protein in the absence of perturbant (Figure 1B). Indeed, substrate-induced difference spectra with the protein prepared in the presence of 20% methanol, ethylene glycol, Me_2SO, or 90% deuterium oxide were identical with those obtained in buffer alone (Figure 1B). Furthermore, the solvent perturbation difference spectra of the protein alone and the protein in the presence of substrate are identical. These results indicate that the exposure of the chromophores is not affected by the presence of substrate. It further shows that the substrate-induced difference spectrum is the result of a direct interaction between the substrate and a tryptophan residue in the active site. Since the different refractive indices of the various solvent mixtures (particularly in the case of deuterium oxide) do not give rise to different values of the galactose-induced absorbance change, it is concluded that the tryptophan residue in the active site is not accessible to deuterium oxide or other perturbants in the presence or absence of substrate.

Galactose Effect on Quenching of Protein Fluorescence Emission by Potassium Iodide. Potassium iodide (KI) has been used as a quencher of protein fluorescence emission; in some cases, addition of substrate results in decreased quenching ("protection") (Lehrer, 1971; Laiken *et al.*, 1972). The quenching of fluorescence emission in the galactose-binding protein in the presence and absence of galactose was examined. In the absence of galactose, KI quenched the fluorescence emission of the protein; a linear response was obtained with increasing amounts of KI (Figure 7). When galactose was present at 1 μM or above in the protein solution, the quenching by KI was decreased, as shown by the increased slope of the $F_0/\Delta F$ *vs.* 1/[KI] plot. Extrapolation to infinite KI concentration indicates that not all tryptophan residues are accessible to quencher either in the presence or absence of galactose. The quenching was entirely due to the I^- ion, as KCl had no effect on the fluorescence emission. Quenching by KI does not shift the wavelength of maximum emission; KI also had no effect on the affinity of the protein for galactose.

Responses of a Mutant Galactose-Binding Protein. A mutant galactose-binding protein which has a 7000-fold reduced affinity for galactose and which does not show the galactose-induced increase in electrophoretic mobility has been de-

[2] E. McGowan, unpublished observation.

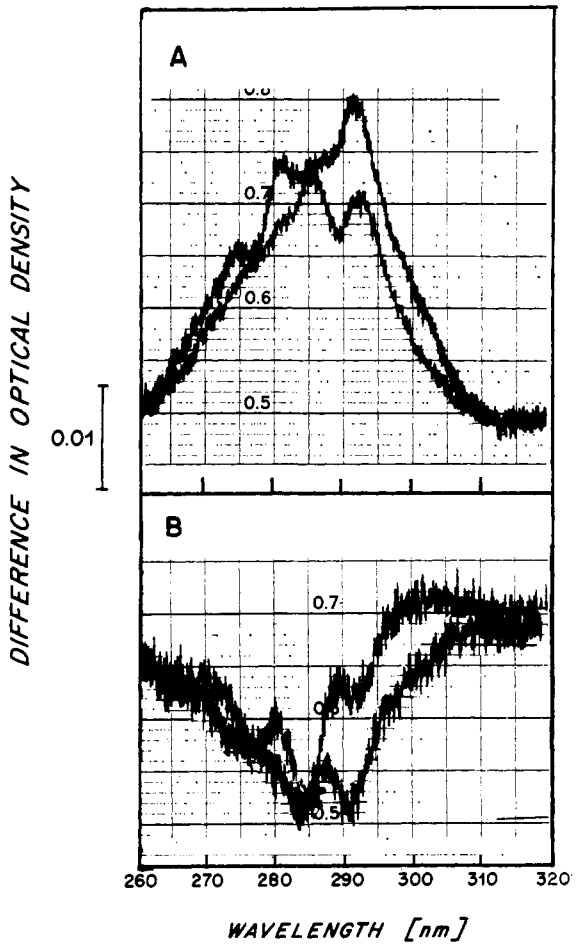

FIGURE 6: Solvent perturbation spectra of the galactose-binding protein in the presence and absence of galactose. (A) Protein (20 μM) was perturbed by 20% (v/v) Me_2SO (lower spectrum), then 0.1 mM galactose was added to the sample solution (upper spectrum). (B) The protein (44 μM) was perturbed by 90% (v/v) D_2O (lower spectrum), then 0.1 mM galactose was added (upper spectrum).

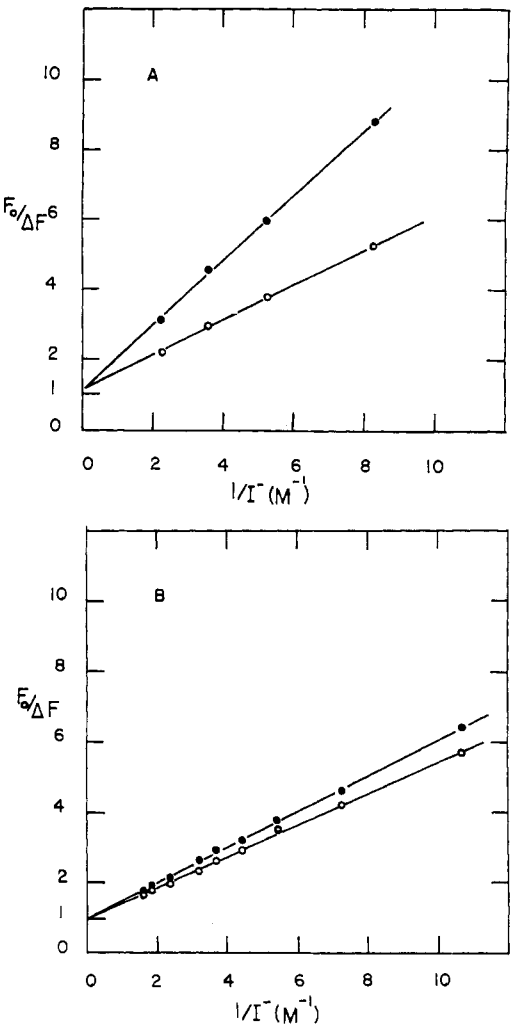

FIGURE 7: Quenching of protein fluorescence emission by KI. To wild-type galactose-binding protein (0.5 μM in 0.01 M Tris-HCl, pH 7.3) in the presence and absence of 10 μM galactose, increasing amounts of 6 M KI were added. A control cuvet obtained increasing amounts of 6 M NaCl. Mutant binding protein was treated identically except that the galactose concentration was 10 mM. 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. The emission wavelength was monitored at 330 nm for the wild-type protein and 336 nm for the mutant protein. Excitation was in both cases 290 nm. (A) Wild-type protein; (B) mutant protein; open symbols, quenching in the absence of galactose; closed symbols, quenching in the presence of galactose.

scribed by Boos (1972). Addition of 10 mM galactose or glucose to a solution of the mutant protein results in altered ultraviolet absorbance. The difference spectra are shown in Figure 8. Difference maxima are obtained at 274, 281, 287, and 293 nm. The two sugars again give rise to similar but not identical spectra; the absorbance above 295 nm is somewhat less pronounced than that in the wild-type protein.

Addition of increasing amounts of galactose to the mutant protein gave a half-maximal effect on the ultraviolet difference spectrum at 1 mM. The mutant protein showed a response to the C-1 hydroxyl group configuration similar to that shown by the wild-type protein—only the peak at 293 nm exhibited a time-dependent increase when fresh α-galactose was added (not shown).

Quenching of fluorescence emission of the mutant protein in the presence and absence of 0.1 M galactose was determined by adding increasing amounts of KI. In contrast to the findings with wild-type protein, the presence of sugar had only a small effect upon the quenching observed with the mutant protein. Also, extrapolation indicates that all tryptophan residues are accessible to KI (Figure 7). No shift in the wavelength of maximum emission was observed.

Discussion

Addition of substrate to the galactose-binding protein results in altered ultraviolet absorbance. The difference spectra obtained resemble solvent perturbation difference spectra of *N*-acetyltryptophan ethyl ester (Herskovits and Sorensen, 1968). These studies together with previous fluorescence studies (Boos *et al.*, 1972) show that the chemical environment of a tryptophan residue is altered upon binding of galactose. Several mechanisms can be proposed. (1) The binding process could induce a refolding of the peptide chain causing an alteration in the exposure of one or more tryptophan residues. (2) The altered ultraviolet absorbance could indicate a direct interaction between the sugar substrate and a tryptophan residue present in the active site. (3) A combination of both processes may occur upon binding.

The galactose and glucose induced difference spectra are similar but not identical; furthermore, the difference spectra of the α and β anomers of galactose are markedly different. These observations indicate that a direct interaction between the sugars and a tryptophan residue takes place, since it is

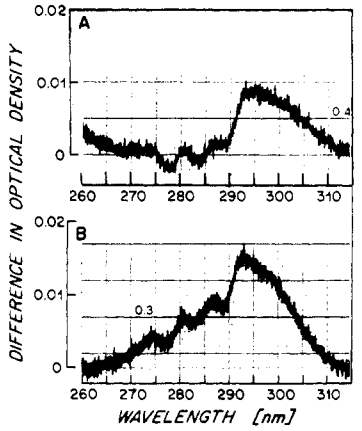

FIGURE 8: Ultraviolet difference spectra of mutant galactose-binding protein caused by galactose and glucose. Experimental conditions as in Figure 1.

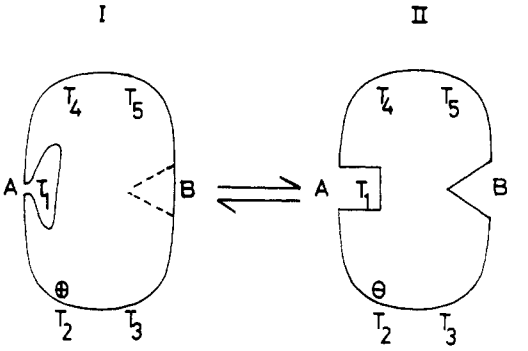

FIGURE 9: Model of the conformational change occurring upon addition of galactose to the protein. (A) High-affinity binding site, active only in state II; (B) low-affinity binding site; T_1, tryptophan in the active site; T_2, T_3, external tryptophan residues accessible to all perturbants; T_2 is located close to an electrical charge, which is different in state I and II; T_4 and T_5, tryptophan residues not accessible to Me_2SO, ethylene glycol or methanol, but accessible to D_2O.

unlikely that such small structural alterations in the substrate could affect the chemical environment of a tryptophan not present in the active site. Solvent perturbation spectroscopy was used in order to investigate the possibility that an additional tryptophan may experience a change in its environment due to a conformational change in the protein. This technique allows the determination of the relative extent of exposure of chromophoric groups in a protein. If the binding of substrate causes a refolding of the peptide chain with a concomitant alteration in the exposure of one or more tryptophan residues, then the solvent perturbation difference spectrum should be altered when obtained in the presence of substrate. Crowder *et al.* (1973) have recently done similar experiments with aldolase. They found that the solvent perturbation difference spectra of this enzyme were markedly different depending on the presence or absence of the competitive inhibitor D-arabinitol 1,5-diphosphate. They concluded that a conformational change occurs upon binding of the inhibitor which results in increased exposure of tryptophan to the solvent at a point removed from the binding site. Similar experiments with the galactose-binding protein described here showed that galactose did not cause any detectable alteration in the solvent perturbation difference spectra produced by a variety of solvents. Thus, external chromophores do not alter their spectral properties upon binding of substrate. It was also found that the difference spectrum produced by the addition of galactose to the protein was not affected to any detectable extent by the presence of various solvent perturbants. This result further substantiates the conclusion that the galactose-induced difference spectrum is due to a direct interaction between the sugar and a tryptophan residue in the active site. Furthermore, since the various solvent mixtures used do not give rise to different galactose-induced absorbance changes, the tryptophan residue in the active site cannot be accessible to the bulk solvent either in the presence or the absence of substrate. It should be noted that since deuterium oxide perturbs the 4 remaining tryptophans the possibility of a buried tryptophan undergoing a change in its chemical invironment can be excluded.

In order to facilitate the understanding of these results, we have prepared a model (Figure 9). Accordingly, the protein exists in two different states, I and II, which are in equilibrium with each other such that in the absence of galactose $II/I \ll 1$. The addition of galactose shifts this equilibrium to the right by combining with the state II conformation. The active-site tryptophan residue (T_1) in state I (binding site A) is inaccessible to the bulk solvent and interacts with substrate only when in state II. Charge differences exist between the two states such that I is more positive than II. Finally, the position of the external chromophoric groups T_2–T_5 is identical in both conformations. Thus the addition of galactose results, primarily, in a perturbation of the active site tryptophan. This residue is not accessible to perturbants in state I, and in state II the galactose prevents the approach of perturbants. Therefore, the solvent perturbation difference spectra are not affected by the presence of substrate. The substrate induced change in electrophoretic mobility (Boos *et al.*, 1972) is accounted for by the charge differences between I and II. Previous studies (Boos *et al.*, 1972) indicate the existence of a second low-affinity binding site (B); however, our present knowledge does not allow a decision on whether or not this site is present in state I.

The fluorescence perturbation data show a protective effect of galactose on quenching produced by KI. Since the solvent perturbation studies indicate that the exposure of the chromophoric groups in the two states is identical and since the active site tryptophan residue is never accessible to perturbants, this protective effect must be the result of something other than the burial of a chromophore. Lehrer (1971) has demonstrated that quenching by iodide can be affected by charged residues near the tryptophan residue in the tertiary structure of the polypeptide. Thus, we conclude that the alteration in surface charge of the protein upon binding of galactose causes the differential quenching of an external tryptophan. Two observations support this conclusion. (i) Fluorescence perturbation studies on the mutant galactose binding protein show only a small protective effect of galactose, paralleled by the observation that this protein does not alter its surface charge upon binding. (ii) Both the galactose- and glucose-induced difference spectra of the wild-type protein show marked positive absorbance above 295 nm. Ananthanarayanan and Bigelow (1969a,b) have studied the difference spectra of tryptophan model compounds and proteins, and report that ΔA_{300nm} values vary depending on change in the electrostatic environment of tryptophan residues. A more negative environment resulted in positive values of ΔA_{300nm}. The mutant protein also shows some positive absorbance above 295 nm, even though no increase in negative surface charge occurs. We interpret this absorbance by an overall red shift of the sugar induced spectrum; indeed, the maximum of this difference spectrum is shifted from 291 to 293 nm.

The results confirm that a conformational change accompanies the binding of substrate to the galatose-binding protein. This changes does not involve a major refolding of the peptide backbone but is rather confined to small alterations that seem to involve only the active site region of the molecule. The substrate-induced fluorescence increase and ultraviolet difference spectra are for the most part a result of a direct interaction between the substrate and a tryptophan residue present in the active site. At least one other tryptophan is involved, however, and this residue appears to lie in the immediate vicinity of an amino acid(s) which develops a more negative charge concomitant with substrate binding. The role of this conformational change in the normal physiological functioning of the galactose-binding protein remains to be established.

Acknowledgments

We thank Dr. Herman Kalckar for his generous hospitality and interest in this work. We thank Dr. Othmar Gabriel for helpful discussions, Dr. Harry Hogenkamp and Dr. H. Isbell for samples of allose and gulose, and Lena Alberico for technical assistance.

References

Ananthanarayanan, V. S., and Bigelow, C. C. (1969a), *Biochemistry 8*, 3717.
Ananthanarayanan, V. S., and Bigelow, C. C. (1969b), *Biochemistry 8*, 3723.
Anraku, Y. (1968a), *J. Biol. Chem. 243*, 3116.
Anraku, Y. (1968b), *J. Biol. Chem. 243*, 3123.
Anraku, Y. (1968c), *J. Biol. Chem. 243*, 3128.
Boos, W. (1969), *Eur. J. Biochem. 10*, 66.
Boos, W. (1972), *J. Biol. Chem. 247*, 5414.
Boos, W., and Gordon, A. S. (1971), *J. Biol. Chem. 246*, 621.
Boos, W., Gordon, A. S., Hall, R. E., and Price, H. D. (1972), *J. Biol. Chem. 247*, 917.
Boos, W., and Sarvas, M. O. (1970), *Eur. J. Biochem. 13*, 526.
Crowder, A. L., III, Barker, R., and Swenson, C. A. (1973), *Biochemistry 12*, 2078.
Edelhoch, H. (1967), *Biochemistry 6*, 1948.
Gornall, A. G., Bardawill, C. J., and Davis, M. M. (1949), *J. Biol. Chem. 177*, 751.
Hayashi, K., Imoto, T., and Funatsu, M. (1963), *J. Biochem. (Tokyo) 54*, 381.
Herskovits, T. T., and Laskowski, M., Jr. (1960), *J. Biol. Chem. 235*, PC56.
Herskovits, T. T., and Laskowski, M., Jr. (1962), *J. Biol. Chem. 237*, 2481.
Herskovits, T. T., and Sorensen, M. (1968), *Biochemistry 7*, 2523.
Laiken, S. L., Gross, C. A., and von Hippel, P. H. (1972), *J. Mol. Biol. 66*, 143.
Lehrer, S. S. (1967), *Biochem. Biophys. Res. Commun. 29*, 767.
Lehrer, S. S. (1971), *Biochemistry 10*, 3254.
Lengeler, J., Hermann, K. O., Unsöld, H. J., and Boos, W. (1971), *Eur. J. Biochem. 19*, 457.
Parnes, J. R., and Boos, W. (1973), *J. Biol. Chem. 248*, 4436.
Rotman, B., and Ellis, J. H. (1972), *J. Bacteriol. 111*, 791.

Purification and Characterization of Histidyl Transfer Ribonucleic Acid Synthetase of *Escherichia coli*†

Frantisek Kalousek and William H. Konigsberg*

ABSTRACT: Histidyl-tRNA synthetase from *Escherichia coli* K12 was purified 820-fold to apparent homogeneity as shown by electrophoresis in polyacrylamide gels at pH 8.5 and in polyacrylamide gels containing sodium dodecyl sulfate. No other aminoacyl-tRNA synthetase activity could be detected. The enzyme has a mol wt of ~85,000 and is a dimer consisting of apparently identical 42,500-dalton subunits. The enzyme has the following characteristics: (a) two molecules of ATP and histidine are bound per molecule of enzyme; (b) the K_m values for ATP and histidine are similar to those found for the substrates of other aminoacyl-tRNA synthetases; (c) histidinol is an effective inhibitor; (d) two sulfhydryl groups per molecule of the histidyl-tRNA synthetase reacted with 5,5′-dithiobis(2-nitrobenzoic acid) and the SH groups are essential for enzymatic activity; (e) a low concentration of trypsin destroys the enzyme activity.

The proper attachment of amino acids to their cognate tRNAs by aminoacyl-tRNA synthetases is one of the crucial steps for ensuring fidelity in the translation of the genetic code. This fidelity depends on the correct recognition of both the amino acid and the corresponding tRNA by the aminoacyl-tRNA synthetase. There seems to be only one such enzyme for each of the 20 amino acids (Lengyel and Söll, 1969). Many of them have been purified and characterized from procaryotic and eucaryotic organisms.

These enzymes have been classified into three groups based on their quaternary structure (Muench and Joseph, 1971). The apparent diversity in quaternary structure among these enzymes has prompted speculation that some of them are involved in other functions of the cell such as regulation of certain biosynthetic enzymes (Calvo and Fink, 1971). The histidyl-tRNA synthetase might be involved in this way since mutants having a lesion in the structural gene for this enzyme show elevated

† From the Department of Molecular Biophysics and Biochemistry, Yale University Medical Center, New Haven, Connecticut 06510. *Received June 25, 1973.* Supported by a research grant from the U. S. Public Health Service (GM 12607).