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Characteristics of the Reversible Heat, Solvent, and Detergent Denaturation of Leucine Binding Protein†

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ABSTRACT: The conformation and binding capacity of leucine binding protein from *Escherichia coli* was measured over a range of temperatures and solvent compositions. At temperatures less than 60° the apparent dissociation constant (K_d) followed a linear Arrhenius relationship and the protein showed no fluorescence changes indicative of conformational changes. Above 60° large increases in K_d were accompanied by quenching and shift of protein fluorescence typical of gross denaturation. Presence of 0.16 mM L-leucine increased the transition temperature by about 10°. The changes were almost completely but slowly reversible. Increases in methanol, ethanol, and dioxane concentration did not produce appreciable changes in binding until relatively high concentrations were reached where pronounced fluorescence changes occurred, except that with dioxane a largely competitive inhibition of leucine binding was noted. Significantly, optical rotatory dispersion measurements indicated 20 vol % dioxane nearly doubled the helical content of the protein with little change in binding capacity. Presence of leucine did not prevent the optical rotatory dispersion changes induced by dioxane. Addition of sodium dodecyl sulfate decreased the total binding capacity and increased K_d. Gel electrophoresis experiments gave no evidence for interconvertible forms with differing binding capacities. These and other results do not give support to active transport models based on conformational changes of binding proteins.

In the past few years, various low molecular weight proteins have been isolated from bacteria, particularly *Salmonella typhimurium* and *Escherichia coli*, which specifically bind certain amino acids, sugars, or inorganic ions. Roles for these proteins as carriers in the active transport of their ligands is suggested by a number of criteria, summarized by Pardee (1968) and by Kaback (1970). Similar considerations have been given by Penrose *et al.* (1970) for leucine binding protein of *Escherichia coli*, studies of which are reported here.

How metabolic energy may be coupled to active transport is unknown. No enzyme activity, modification in binding, or other response to the presence of ATP has been found associated with these proteins. One possibility for participation of the binding proteins in active transport is that energy-linked conformation changes are correlated with the release of the ligand. Support for such a possibility comes from the detection by Boos and Gordon (1971) of interconvertible forms of galactose binding protein. These forms had properties under physiological conditions like native protein, but had pronounced differences in binding capacity.

Such interconvertible forms have not been reported for other binding proteins. Penrose *et al.* (1970) found no evidence of ligand-induced conformation changes as assessed by optical rotatory dispersion (ORD) and fluoresence probes. They did demonstrate that leucine binding protein will undergo reversible denaturation with respect to activity and physical properties when treated with urea or guanidine hydrochloride, or exposed to high temperature or extremes of pH. The conditions they used were such that gross denaturation of the binding protein had likely occurred, that is, the disruption of many hydrophobic interactions and hydrogen bonds, followed by removal of the denaturing condition and regaining of the native protein properties. For example, the binding protein was exposed to 100°, then cooled, and assayed at room temperature. It is likely, however, that any conformation changes associated with the release of the bound substance as part of the transport process would be quite limited. A slight displacement of interacting portions of the molecule, or of even one or a few groups might suffice. Extensive changes characteristic of gross denaturation would not be necessary for changes in binding activity.

The present paper presents data on the binding capacity of leucine binding proteins over graded temperature ranges and with various solvent compositions, with use of protein fluorescence and optical rotatory dispersion as criteria for conformation changes and denaturation.

Materials

Crystalline leucine binding protein was prepared as described by Penrose *et al.* (1968) but using the osmotic shock procedure of Anraku (1968) to allow the preparation of larger amounts of protein. The protein concentration was determined using a value of 0.65 as the optical density of 1 mg/ml at 280 nm and a molecular weight of 35,000 (Penrose *et al.*,

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1968). EDTA (1 mM) was added to retard bacterial growth and the protein was stored at 4°. L-[^{14}C]Leucine in 0.01 M HCl was purchased from Schwarz-Mann Radio-Chemical Inc.

Ammonium ANS[1] was purchased from Eastman and was recrystallized twice from water as the Mg salt (Weber and Young, 1964). Concentrations were determined using an extinction coefficient of 4950 cm^{-1} M^{-1} at 350 nm (Weber and Young, 1964).

Methanol and dioxane were Matheson, Coleman & Bell Spectroquality and Scintillation Quality reagents, respectively. Just before use the dioxane was chromatographed on activated alumina to remove peroxides as described by Dasler and Bauer (1946). In one experiment, the dioxane was further purified by treatment with HCl and then sodium metal (Fieser and Fieser, 1967). It was then fractionally distilled in 3-ft Vigreaux column. Sodium dodecyl sulfate was a purified grade from Sigma Chemical Co. Other chemicals were reagent grade.

Methods

Binding assays were carried out by equilibrium dialyses in Lucite chambers separated by a membrane prepared from Visking dialysis tubing. The membranes were placed between rings prepared from parafilm or polyethylene film which served as gaskets to prevent leaks. Protein solution (0.2 ml) was dialyzed *vs.* 0.2 ml of a solution containing L-[^{14}C]leucine. After equilibrium was reached (in a time determined for different temperatures and solvent compositions), 0.1-ml samples of each chamber were counted for radioactivity in 10 ml of Bray's solution (Bray, 1960) in a Packard scintillation counter. In some cases the leucine was added to the protein solution to check whether the equilibrium point was attained.

In binding experiments where high concentrations of organic solvents were used a buffer was omitted. Solutions before the addition of solvent were adjusted to a pH of 6.0–7.0 to neutralize the small amounts of HCl in which the L-[^{14}C]leucine was stored. It should be noted that Penrose *et al.* (1970) found no effect of pH on the binding of leucine over the pH range 4–9.

Fluorescence studies were carried out with an Aminco-Bowman spectrofluorometer fitted with a temperature-controlled cell holder. In experiments in which temperature was varied, the temperature in the sample was recorded directly with a calibrated glass-enclosed thermistor immersed in the sample (1-ml volume) out of the light path. At high temperatures the protein was found to be sensitive to photooxidation and an apparent irreversible decrease in fluorescence intensity was observed when the protein was cooled. This problem was minimized by opening the excitation slit only momentarily to record the fluorescence intensity. Light stability was checked by measurement of a standard from time to time. Spectral data reported are observed intensities and wavelengths and are not corrected for instrumental characteristics.

Optical rotatory dispersion studies were carried out with a Cary Model 60 recording spectropolarimeter. Solutions were filtered through a solvent-resistant (UR type) Millipore filter directly into the cell. Blanks were run before and after each sample. L-Leucine, when added to the samples, was also added to the blank to correct for the optical rotation due to leucine itself. Data are reported as the mean residue rotation at 233 nm, $[m']_{233} = [\alpha M_0/cl][3/(n_{233}^2 + 2)]$, where α is the observed rotation, M_0 is the mean residue molecular weight (taken as 115), c is the protein concentration in g/100 ml, l is the light path in decimeters, and n_{233} the refractive index at 233 nm. An approximate value for the Lorentz correction was calculated for dioxane utilizing the refractive indices of H_2O and of dioxane at the sodium D line (Fasman, 1963). A linear interpolation was made between values of the pure solvents to obtain values for the dioxane–H_2O mixtures.

Polyacrylamide gel electrophoresis was carried out by the method of Davis (1964) or the method of Boos and Gordon (1971). Sample and stacking gels were not used in the former procedure but the sample was layered over the gel in a 20% sucrose solution. The latter method was modified for use with gels polymerized in 5-mm diameter glass tubes rather than in 4-mm thick slabs. The persulfate concentration was reduced by one-half to slow polymerization time. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed as described by Boos and Gordon (1971), with use of 1.8–9 μg of protein in 10 μl. Gels were stained for protein with Coomassie Blue (Chrambach *et al.*, 1967).

TABLE I: Effect of Temperature on the Binding of Leucine by Leucine Binding Protein.[a]

T (°C)	Estimated K_d[b]
5.0	0.36 ± 0.02 (6)
30.0	0.81 ± 0.22 (4)
50.0	1.28 ± 0.20 (4)
59.2	5.95 ± 1.28 (3)
64.5	26.10 ± 10.70 (11)

[a] Binding activity was determined by equilibrium dialysis. Leucine binding protein concentration was 0.134 mg/ml (at 64.5°, 0.195 mg/ml). Initial leucine concentration, 1.06 μM. All solutions contained 0.04 M potassium phosphate (pH 6.9). Equilibration time varied from 20 hr at 5° to 3 hr at 64.5°. Other details are given in the section on Methods. [b] Average K_d ± one average deviation. The numbers in parentheses are the number of determinations.

Results

Effect of Temperature on Binding Activity. Values of K_d, the dissociation constant of the protein–leucine complex, observed at different temperatures are given in Table I. An Arrhenius plot of these data is shown in Figure 1. Over the range of 5–59°, the plot is linear indicating a normal temperature dependence of an equilibrium process with a constant activation energy of 5.4 kcal/mole. Above 59°, the dissociation constant increases more rapidly suggesting that conformation changes in the protein are now contributing to the weakening of the binding. The average value for the activation energy between 59 and 64° is calculated to be 61.9 kcal/mole.

The effect of temperature on binding was reversible. Binding protein was heated at 65° for 3 hr, the time to reach equilibrium under the conditions of the equilibrium dialysis assay at this temperature, and then binding activity measured at 5°. No loss of binding activity compared to control was noted.

Large irreversible losses of activity at a temperature of 70° prevented the determination of a binding constant at this temperature.

[1] Abbreviation used is: ANS, 1-anilinonaphthalene-8-sulfonic acid.

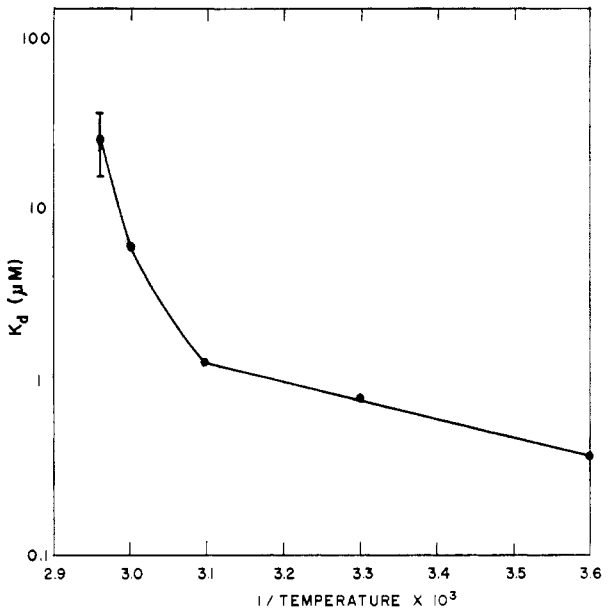

FIGURE 1: Reciprocal plot of the effect of temperature on the K_d for leucine binding protein. Brackets indicate the average deviation of the determined K_D. Data are taken from Table I.

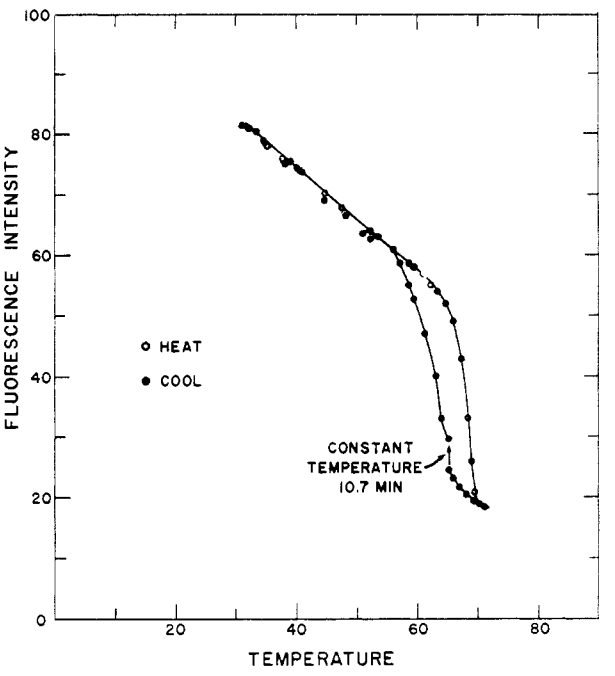

FIGURE 2: Effect of temperature on fluorescence of leucine binding protein. Fluorescence intensity at 341 nm and temperature were determined as described in the section on Methods. The rate of temperature change was 0.25–2.0 deg/min. The 1-ml samples contained 0.02 mg of leucine binding protein and 0.16 mM leucine in 0.04 M potassium phosphate buffer at pH 7.0

Effect of Temperature on Leucine Binding Protein Fluorescence. In order to correlate changes in the dissociation constant of the leucine binding protein with structural changes in the protein, the effect of temperature of the protein fluorescence was studied. It has been observed that deviations from a linear or near linear decrease in fluorescence intensity with increasing temperature will occur if there are structural changes affecting the environment of tryptophan residues of the protein. The fluorescence of proteins has been shown by Teale (1960) and by Weber (1960) to be primarily due to tryptophan fluorescence. Thermally induced structural changes will be observable as changes in the slope of the quenching curve or the occurrence of irreversible or time-dependent intensity changes (Steiner and Edelhoch, 1961, 1963). Penrose *et al.* (1968) have shown that 12 moles of tryptophan are present per mole of leucine binding protein. Assuming a near random distribution of these residues in the protein, large fluorescence changes would be indicative of gross conformational changes in the protein.

Leucine binding protein showed an emission maximum of 341 nm at 25° when excited by light at 292 nm. The temperature dependence of the fluorescence in the presence of 0.16 mM L-leucine is shown in Figure 2. Up to 60°, the quenching curve shows a monotonic decrease in fluorescence intensity which is completely reversible and is characteristic of tryptophan in solution (Steiner and Edelhoch, 1963).

Above 60°, a sharp decrease in fluorescence intensity occurs as well as a shift in emission maximum to 356 nm. Above 70° the rate of intensity decrease with temperature increase is approximately the same as below 50°. The quenching of protein fluorescence and shift to a longer wavelength maximum is consistent with an unfolding of the molecule with exposure of tryptophan residues to a more polar environment. The transition was largely though not entirely reversible. A slow reversibility is indicated by the displacement of the transition in the cooling curve to lower temperatures compared to the heating curve.

In the absence of added L-leucine, the midpoint of the thermally induced transition was lowered from 68–70 to 59–60°. Thus the binding of leucine confers considerable stability to the native configuration. In the presence as well as the absence of leucine the fluorescence at a given temperature during cooling tended to be less than that at the same temperature during heating. The deviation was more marked in the presence of leucine. A time lag in regaining of fluorescence was responsible. For example, if the sample was cooled to 65° then the temperature held constant, a slow increase in fluorescence was observed.

Effect of Temperature on ANS Fluorescence. Further study of thermally induced conformation changes could be carried out by observing the fluorescence of ANS bound to leucine binding protein. While ANS fluoresces poorly in water solution, in nonpolar environments the quantum efficiency can greatly increase with shift of the emission maximum to lower wavelengths (Edelman and McClure, 1968). During denaturation of many proteins hydrophobic regions are exposed to solution and reveal their presence by enhancement of ANS fluorescence (Edelman and McClure, 1968). Upon the addition of 1.9 μM leucine binding protein to 0.124 mM ANS in 0.04 M potassium phosphate (pH 6.9), only 2% enhancement of ANS fluorescence was observed indicating that, under these conditions, tight binding sites with high quantum efficiency were absent. Upon heating up to 55° the fluorescence intensity changes, which were completely reversible, were accountable by an effect of temperature of ANS fluorescence in the absence of protein. In the absence of protein a 17% increase in ANS fluorescence was obtained by heating from 30 to 63°. Above 55° large increases in fluorescence occurred concomitant with shifts of emission maximum to lower wavelengths. The fluorescence intensity at 63.4° was 185% greater than that observed at 23°, and with an emission maximum shift from 513 to 500 nm. These data are in accord with the conclusions from the effect of temperature on tryptophan

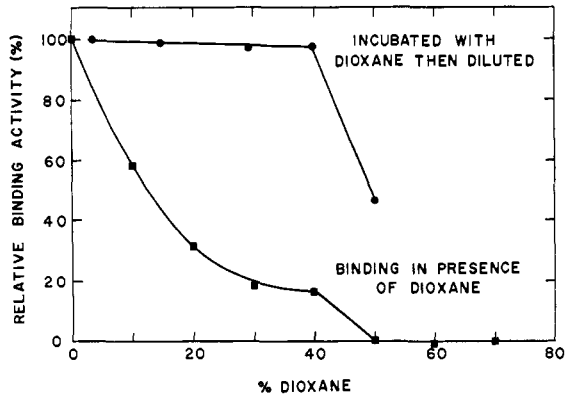

FIGURE 3: Effect of dioxane on binding activity and stability of leucine binding protein. Duplicate samples of protein, 68 μg/ml, were equilibrated with 2.2 μM L-[^{14}C]leucine for 26 hr at 4°. Both solutions contained the indicated dioxane concentrations and 0.05 M KCl. To test stability under these conditions, leucine binding protein (1.0 mg/ml) was incubated in 0.1 ml for 28 hr at 4° at the indicated concentration of dioxane and 0.05 M KCl. The solutions were then diluted 1:15 to 3.3 vol % dioxane and 0.05 M KCl and duplicate samples assayed for binding activity by equilibrium dialysis. Results are plotted as per cent leucine bound relative to the control lacking dioxane.

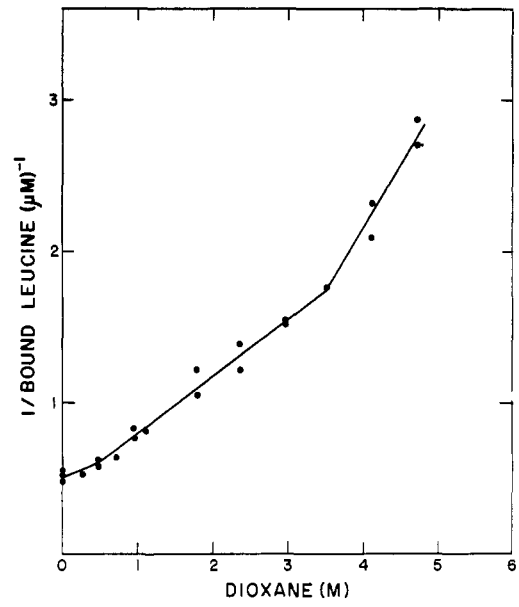

FIGURE 4: Reciprocal plot of leucine bond *vs.* dioxane concentration. Conditions were as in Figure 3, but the leucine binding protein concentration was 0.21 mg/ml and temperature 7°.

fluorescence: that above 55° a major unfolding of the protein occurs with exposure of hydrophobic regions to solution.

Somewhat surprisingly the fluorescence intensity changes did not reverse when the protein was cooled but rather increased almost twofold with a further shift of the emission maximum to 485 nm. It is possible that the ANS binding sites permanently created by heat treatment may represent a small portion of the total protein present, possibly a minor impurity which is irreversibly denatured. The present data do not allow a firm answer to this question. However, binding activity is completely reversible.

Solvent Effects on Binding Activity. The effect of varying concentration of dioxane on binding activity is shown in Figure 3. Approximately 11 vol % dioxane was required for 50% inhibition of binding activity. Similar effects were observed with methanol and ethanol, except higher solvent concentrations were required to prevent binding and the biphasic tendency of the dioxane effects was not observed. Approximately 38 vol % ethanol or 62 vol % methanol was required for 50% loss in binding activity. The three solvents inhibited the activity at concentrations that roughly paralleled the dielectric constant of the solvent.

The inhibitory effects of the solvents were reversible to varying extents, depending on solvent and solvent concentration as indicated by experiments in which the protein was incubated with the solvent, diluted and then assayed. At higher solvent concentrations, tendency for irreversible effect on binding was noted.

The possibility that the inhibition by dioxane was due to a trace impurity was rendered less likely by purification of the solvent by distillation as described in the section on Materials. The purified solvent showed quite similar inhibition effects to the unpurified material.

The inhibition by dioxane was measured at various leucine concentrations to determine whether inhibition was due to loss of binding capacity or to an increased K_d. At a concentration of 10 vol % dioxane, leucine was found to be competitive with dioxane with an increase of K_d from 1.2 to 2.1 μM (Figure 4). The biphasic curve for dioxane inhibition (Figure 3) suggests that two processes are involved in dioxane inhibition, one reversible phase occurring below 40 vol % dioxane and the other less reversible phase above 40 vol % dioxane.

Conceivably dioxane, although not grossly similar to leucine in structure, could bind weakly at the leucine site because both compounds contain hydrophobic residues in the form of chains of methylene groups. The concentration dependence of dioxane inhibition was thus studied more closely with particular emphasis on the low concentrations. If dioxane were a simple competitive inhibitor of leucine binding, a linear relationship of reciprocal leucine bound *vs.* dioxane concentration would be observed. On the other hand, if a perturbation of the protein structure by dioxane were responsible for the inhibition, such an effect would probably require sufficient dioxane present to change solvent characteristics appreciably or to give binding of many dioxane molecules per protein molecule. A departure from a linear relationship indicative of cooperativity might be observed as with methanol and ethanol which inhibit only at concentrations above 30–40 vol %. An apparent linear relationship of reciprocal of bound leucine *vs.* dioxane concentration was observed over the range 4–30 vol % dioxane (0.43–3.5 M). As shown in Figure 5 it is evident that inhibition by dioxane is present at very low concentrations (<4%). Over the range from 0 to 16 vol % dioxane a linear relationship is observed, whereas a curve concave upwards would be expected for simple competitive inhibition. Simple competitive inhibition of dioxane *vs.* leucine appears insufficient to explain the data.

Effect of Solvents of Leucine Binding Protein Fluorescence. Effects of solvents on protein conformation were assessed by fluorescence and optical rotation measurements. The effect of increasing dioxane and ethanol concentration on the fluorescence of leucine binding protein and a model compound, *N*-acetyltryptophanamide is shown in Figure 6. Decreasing polarity of solvent increases the fluorescent yield of *N*-acetyltryptophanamide. Both solvents have a very small effect on the fluorescence of leucine binding protein indicating very little

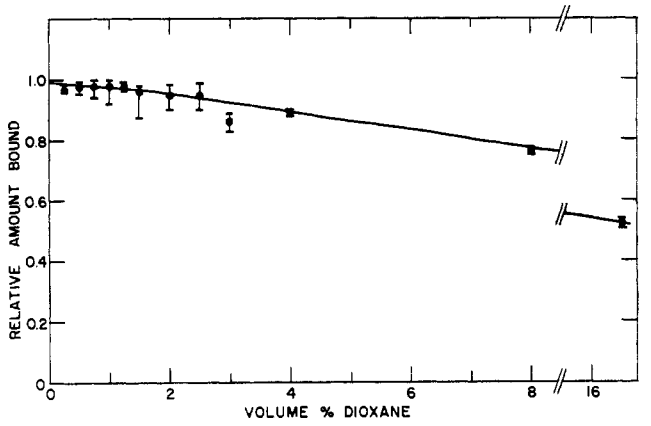

FIGURE 5: Effect of low concentrations of dioxane on leucine binding. Conditions are the same as those of Figure 4. Data from several experiments with a total of three to nine samples at each concentration were averaged. Brackets show the extent of variation.

exposure of the tryptophan residues to the protein to solution. Conformational changes affecting these residues are indicated at 40–50 vol % solvent where the emission maximum is shifted to higher wavelengths. The broadening and the assymmetric shape of the peak indicate that the tryptophan residues were in at least two distinct environments above 40 vol % organic solvent. These data indicate that appreciable changes in the dielectric constant of the medium may occur without large changes in the conformation of the protein as revealed by changes in the fluorescence of the tryptophan residues.

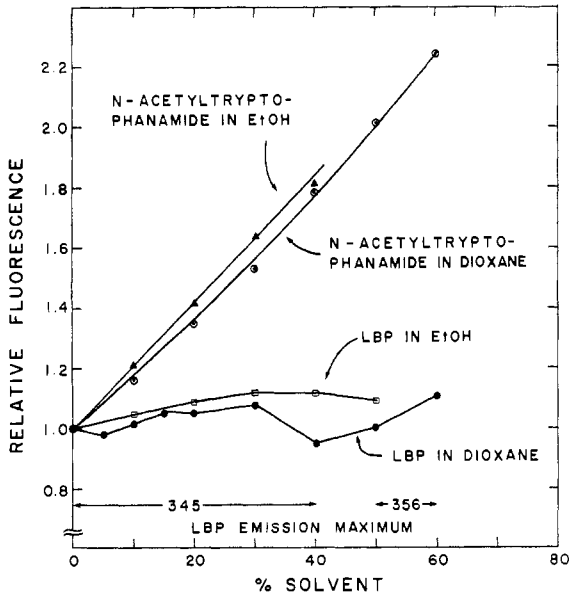

FIGURE 6: Effect of dioxane and ethanol on fluorescence of leucine binding protein and *N*-acetyltryptophanamide. The protein was diluted to 0.02 mg/ml in ethanol solutions or 0.05 mg/ml in dioxane solutions just before spectra were recorded. Samples contained 0.05 M KCl in addition to the indicated solvent concentration. *N*-Acetyltryptophanamide concentration was 10 μM. Excitation was at 290 nm and 25°. None of the intensity values were time dependent at the time spectra were recorded (approximately 5 min after dilution of the protein). Values are corrected for intensities of a fluorescent standard determined before and after sample spectra were recorded.

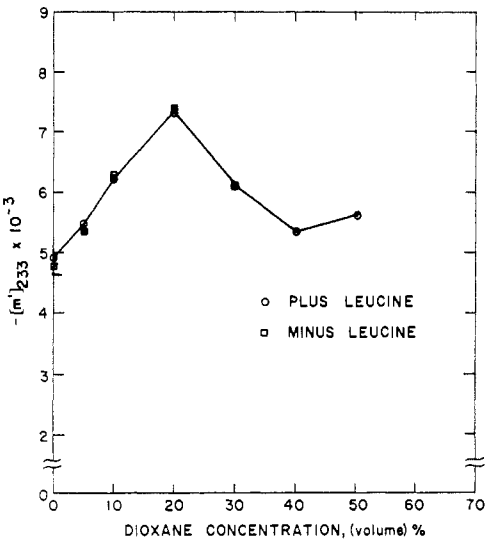

FIGURE 7: Effect of dioxane on the ORD of leucine binding protein. Leucine binding protein was diluted to 50 μg/ml just before spectra were recorded. Solutions also contained 0.05 M KCl. Other details are given in the section on Methods. LBP designates leucine binding protein.

Concentrations of leucine up to 10 mM did not affect the fluorescence intensity of the leucine binding protein or the the solvent effects on fluoresecnce emission.

The shift in emission maximum in the presence of ethanol correlates with the concentration range where ethanol shows inhibition of binding activity and in the presence of dioxane with the second phase of dioxane inhibition leading to complete loss of binding activity occurs (Figure 3).

Effect of Dioxane on ORD of Leucine Binding Protein. As shown in Figure 7, increase in dioxane concentration up to 20 vol % decreased the optical rotation at 233 nm, with increase in rotation above 20 vol % dioxane. The maximum of optical rotation at 233 nm has been correlated with the extent of α-helical content of proteins. Assuming a mean residue rotation value at 233 nm of $-16{,}200$ for 100% α helix and -2200 for the disordered structure (Jirgensons, 1969), the protein helical content increased from 19 to 37% over the range 0–20% dioxane. Addition of 1 mM L-leucine had less than 3% effect on the ORD throughout the dioxane concentration tested. Although 1 mM leucine will overcome the inhibitory effect of 10 vol % dioxane on binding (see Figure 4), it did not affect the conformation change observed by ORD. This suggests that the binding site for leucine was not in the region of the molecule where the α-helical content was changing.

Reversible Inhibition of Binding by Sodium Dodecyl Sulfate. The effect of sodium dodecyl sulfate on binding activity is shown in Figure 8. Reversibility was tested by incubation at various concentrations followed by dilution. The nature of the inhibition in the reversible region was explored further by measurement of the leucine concentration dependency of binding at various dodecyl sulfate concentrations. Figure 9 shows a series of plots of the reciprocal of leucine bound *vs.* the reciprocal of free leucine at various sodium dodecyl sulfate concentrations. The intersection of the curves on the abscissa equals $-1/K_d$ and on the ordinate equals $1/n[P]$, where [P] is the total protein concentration. Table II lists values of n and K_d determined from these plots. Sodium dodecyl sulfate increases the apparent dissociation constant and decreases the total number of binding sites. Because only one binding

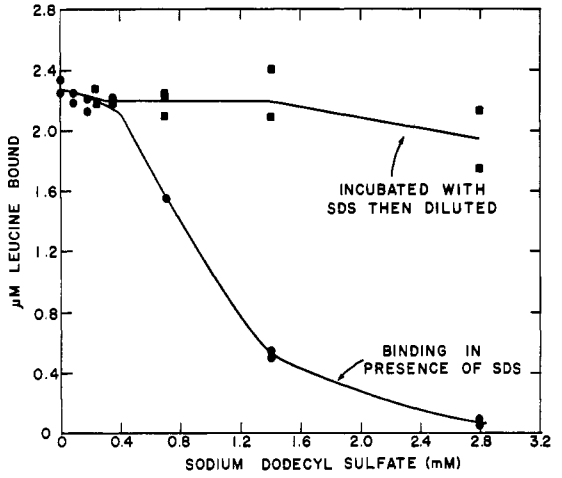

FIGURE 8: Effect of sodium dodecyl sulfate (SDS) on boinding activity. Solutions for equilibrium dialysis contained 0.05 M Tris-Cl (pH 7.15) and the indicated sodium dodecyl sulfate concentrations. The leucine binding protein concentration was 0.196 mg/ml and L-[^{14}C]-leucine was 2.6 μM. Equilibration was for 19 hr at 5°. For reversibility studies, leucine binding protein, 2.56 mg/ml, was incubated 19 hr with the indicated concentrations of sodium dodecyl sulfate at 4°. The samples were then diluted 12.5-fold to 0.006% sodium dodecyl sulfate and assayed as above.

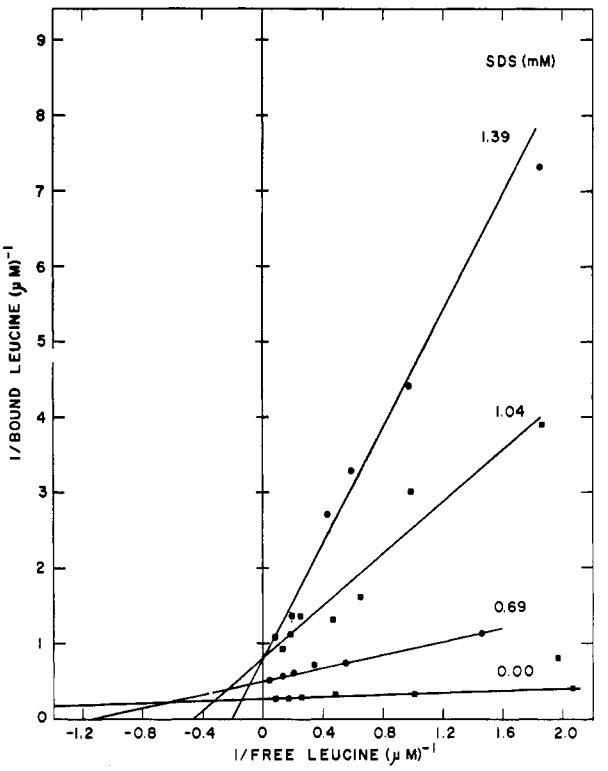

FIGURE 9: Effect of sodium dodecyl sulfate on binding at variable leucine concentrations. Data are plotted as a reciprocal plot of leucine bound *vs.* free leucine. Solutions for equilibrium dialysis contained 0.05 M Tris-Cl (pH 7.15) and the indicated sodium dodecyl sulfate concentrations. The protein concentration was 3.66 μM.

site is present in each native protein molecule, a portion of the protein has sufficiently weak binding to be undetectable under these conditions and the rest has a higher dissociation constant than the native molecule. Because the reciprocal plots are linear, there must exist discrete populations of molecules with these properties rather than protein molecules with a continuous distribution of K_d's.

The pronounced inhibition of binding activity by sodium dodecyl sulfate occurs largely below the critical micelle concentration, approximately 1.3 mM at 0.1 ionic strength and 20° (Reynolds and Tanford, 1970). Also, the monomeric form rather than the micellar form of sodium dodecyl sulfate appears to bind to proteins (Reynolds and Tanford, 1970).

Other Tests for Interconvertible Forms. Evidence favoring two interconvertible forms of the galactose binding protein separable by polyacrylamide gel electrophoresis (Boos and Gordon, 1971) prompted examination of preparations of leucine binding protein for a similar phenomenon. The preparations showed one major component and only a trace of a slightly more rapidly migrating protein. The amount of the second component varied from one preparation to another but was at least than 5% of the major band in intensity. In some cases the minor component was barely detectable.

Recrystallization of one preparation showing two components did not result in significant reduction in the amount of the minor component present. This preparation was then further investigated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Boos and Gordon, 1971). Samples of protein were incubated for 1 hr in a solution containing 2% sodium dodecyl sulfate and 5 mg/ml of dithiothreitol as described by Boos and Gordon (1971). In some experiments the dithiothreitol was omitted. Two components present in the same relative concentrations as in the absence of the detergent were always found. In similar experiments of Boos and Gordon (1971) with the galactose binding protein only one band was noted when electrophoresis was carried out in the presence of sodium dodecyl sulfate but two when the detergent was omitted. The demonstration of a minor component with the leucine binding protein preparations even in presence of detergent and with exposure to dithiothreitol gives good evidence that interconvertible forms of the same protein are not present. In addition, in contrast with the results obtained with the galactose binding protein (Boos *et al.*, 1971) electrophoresis in the presence of the ligand did not affect the rate of migration of the leucine binding protein nor the relative amounts of protein in the two bands observed.

Discussions

The leucine binding protein is representative of an interesting group of proteins whose mode of biological action in active transport is not clear. The changes in binding activity of the protein with conditions that induce conformation change and

TABLE II: Values of K_d and n at Different Sodium Dodecyl Sulfate Concentrations.[a]

Sodium Dodecyl Sulfate (mM)	K_d (μM)	n
0.00	0.34	1.07
0.69	0.88	0.54
1.04	2.12	0.34
1.39	5.00	0.34

[a] Values of K_d and n were determined from the slopes and intercepts of lines in Figure 9.

denaturation are of interest in their own right. They may be of additional value, however, in relation to suggestions that active transport involves conformational changes associated with marked differences in binding affinity for the transported substance. More specifically, the usual suggestion is that an energy-consuming reaction induces a conformational change in the binding protein that causes release of the transported ligand to the cytoplasm, even though the cytoplasmic concentration of the ligand considerably exceeds the extracellular concentration.

Changes in binding affinity for substrates are well known for various allosteric enzymes. In all instances, the changes appear to involve subtle interactions between subunits of multisubunit proteins. Change in one subunit may induce critical changes in orientation at the active site of another subunit with little change in gross structure of each. The binding proteins appear to consist of single polypeptide chains. If such a protein were designed to show readily reversible changes in ligand affinity, the molecular arrangement may be such that relatively large sections of the molecule could move so as to critically affect orientations at the binding site, but without gross structural changes in the large sections. Were this the case, it appears possible that agents or conditions capable of producing denaturation, when applied in a manner far less than that necessary to produce gross denaturation, might induce conformation changes causing decrease in ligand affinity. Such intriguing possibilities were one of the motivating factors for the present studies. Perhaps the most important feature of our and other related data is the failure of various denaturing agents to induce any decisive changes in binding affinity under conditions short of those necessary for gross denaturation.

With respect to temperature effects, the data show that only relatively small changes in binding affinity occur at temperatures below those where gross denaturation occurs. At the temperatures where marked loss of binding capacity occurs (near 60°) extensive changes in protein conformation occur. This is shown by the high energy of activation for the change and by the marked change in fluorescence yield and emission maximum for the protein. In addition, the slow reversal of the change when cooling is initiated (Figure 2) is not in accord with expectations for a conformation change associated with active transport. One would expect any such change to be rapidly reversible. The ability of leucine to elevate the temperature for gross denaturation, although of interest, does not add to the possibility that a conformation change is associated with transport.

The changes in binding capacity with graded increase in concentrations of ethanol, methanol, or dioxane are small, and, again, marked changes in binding capacity do not occur until a condition is reached where extensive denaturation occurs as revealed by fluorescence emission shifts. This is particularly the case for the ethanol effects. The intriguing biphasic effects of dioxane on the binding capacity as noted in Figure 3 could be taken as an indication of a limited conformational change at lower dioxane concentrations associated with considerable loss in binding capacity. However, another alternate possibility exists, namely that the effect of dioxane is largely the result of a competition with leucine for the binding site. Although low concentrations of dioxane have slightly less effect than predicted from a strictly competitive relationship (Figure 5), the data of Figure 4 point largely to a competitive interaction.

A competitive effect of dioxane on leucine binding is in harmony with other observations. Decreases in substrate affinity in the presence of low concentrations of organic solvents have been observed with a number of enzymes. In the cases of pepsin and α-chymotrypsin (Tang, 1965; Applewhite *et al.*, 1958) the effect has been shown to be due to competition of solvent with substrate for a hydrophobic binding site. Glazer (1970) has called attention to the high frequency with which nonpolar compounds unrelated to the substrate in structure bind at the active site of enzymes.

The data of Herskovits *et al.* (1970) indicate that high concentrations of organic solvents denature by changing the conformation of the protein to one of greater α-helical content. With three representative globular proteins they observed 50% denaturation, as determined by various physical parameters, at 29–51 vol % methanol and 22–42 vol % ethanol. Higher concentrations of methanol and ethanol were required to disrupt 50% of the binding activity of the leucine binding protein (62 and 38 vol % solvent, respectively). In addition, these values would have to be considered a lower limit since binding was not measured at saturating leucine concentration. Clearly the leucine binding protein appears unusually stable to organic solvent denaturation.

The effects of dioxane on the ORD in the presence and absence of leucine (Figure 9) illustrate another important point. Although the changes in ORD are indicative of considerable change in the helical content of the protein, such change is independent of whether or not leucine is bound to the protein. The concentration of leucine used was such to saturate the binding site even up to 40 vol % dioxane. These findings are important as they indicate that considerable conformation change can occur in portions of the protein molecule without disruption of the binding capacity.

The changes in binding strength and capacity noted upon addition of the detergent, sodium dodecyl sulfate, show two distinct effects. At all detergent concentrations in the region of inhibition, binding activity of a fraction of the protein is destroyed. The total concentration of sodium dodecyl sulfate required to give complete binding inhibition is somewhat above the free detergent concentration of 0.5 mM cited by Fish *et al.* (1970) as necessary to disrupt and make uniform the hydrodynamic properties of a number of proteins. The effect is thus attributable to gross denaturation, and, as the binding activity is recovered upon removal of the detergent, represents another case of reversible denaturation of the leucine binding protein. The data also indicate a weak competition of dodecyl sulfate with leucine, although the possibility of indirect effects cannot be eliminated by the present data.

Our data are in harmony with and extend the observations of Penrose *et al.* (1970) on the structure of the leucine binding protein. Their studies of the rate of iodination, antigenicity and optical rotation of the protein in the presence of denaturing reagents such as urea and guanidine hydrochloride indicate that gross conformation changes are necessary to destroy binding.

As mentioned earlier, Boos and Gordon (1971) showed that their preparations of galactose binding protein existed under physiological conditions in two forms of different affinity separable by gel electrophoresis. The concentration of ligand determined the fraction of protein in the form of low affinity (Boos *et al.*, 1971). Our electrophoresis experiments with the leucine binding protein showed the presence of only a trace of a second component which is probably an impurity. L-Leucine concentration had no effect on the electrophoretic pattern. Gel electrophoresis in the presence of detergent after exposure to dithiothreitol showed two components,

whereas interconvertible forms should give a single component under these conditions. Our binding experiments did not reveal the presence of an appreciable concentration of a form with low affinity for leucine. Within an experimental error of about 10% all the binding by leucine binding protein could be explained by one binding site per molecule with a K_d of less than 1 μM. Thus we do not find any evidence for distinct conformational states as reported by the galactose binding protein.

Our results obviously do not rule out the possibility of small conformational disruptions of leucine binding protein that decrease the affinity for leucine to the extent necessary to account for active transport. Attempts to attain *in vitro* conditions to mimic a subtle *in vivo* conformation change that might occur in a membrane can obviously fail to disclose such a property even if it exists. But the tightness of the binding of leucine under a variety of stress conditions dampens our enthusiasm for hypotheses of active transport based on conformational change of binding proteins. An alternate possibility involving membrane conformational changes without any change in affinity of the binding protein for its ligand is currently under consideration (Boyer and Klein, 1972).

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Binding of Actin to Heavy Meromyosin in the Absence of Adenosine Triphosphate†

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ABSTRACT: Using the analytical ultracentrifuge, the binding of actin to heavy meromyosin (HMM) in the absence of ATP was studied under varied conditions of ionic strength and temperature. Under all conditions studied, the binding ratio was found to be 2 moles of actin monomer/mole of HMM suggesting that both heads of the HMM bind simultaneously to the actin. Furthermore under all conditions studied, the dissociation constant was found to be less than 6×10^{-7} M. This shows that at an ionic strength of 0.1 M, the binding in the absence of ATP is more than 200-fold stronger than the binding in the presence of ATP as estimated from kinetic measurements which in turn suggests that ATP has a remarkably strong influence on the binding of actin to HMM.

It is now generally established that molecules of myosin and its tryptic digestion product, heavy meromyosin (HMM)[1] each contain two active sites or "heads" (Stracher and Dreizen, 1966; Slayter and Lowey, 1967; Lowey *et al.*, 1969) whereas actin monomers are single polypeptide chains (Rees and Young, 1967). On this basis it might be expected that each myosin or HMM molecule would combine with two monomers in the F-actin filament. However, despite numerous studies on the binding of actin to myosin or HMM, it is still

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[1] Abbreviation used is: HMM, heavy meromyosin.