Circular Dichroic and Optical Rotatory Dispersion Spectra of the Threonine-Inhibited Aspartokinase–Homoserine Dehydrogenase of *Escherichia coli* K 12. Effects of Ligand Binding and Protein Denaturation*

Henry d'A. Heck and Paolo Truffa-Bachi†

ABSTRACT: The circular dichroic and optical rotatory dispersion spectra of the allosteric protein, aspartokinase I–homoserine dehydrogenase I, of *Escherichia coli* K 12, and of its potassium, threonine, aspartate, and NADPH complexes have been measured from 600 to 210 nm. Denaturation of the protein in 6 M guanidinium chloride shows the spectra to be strongly dependent on conformation. Evidence is obtained from both types of spectra that the protein is not randomly coiled in 6 M guanidinium chloride unless disulfide bonds are broken. In the native protein, two types of effects arise from ligand binding. Noncooperative binding of aspartate or threonine leads to changes in circular dichroic bands only at wavelengths greater than 267 nm, which are most readily interpreted in terms of a specific interaction of the ligand with a protein chromophore, or of a local conformational change. In contrast, the cooperative binding of threonine, aspartate, or potassium causes larger perturbations of circular dichroic bands over a broader region of the near ultraviolet spectrum. Threonine and potassium also cause changes in far ultraviolet bands. NADPH binding results in the appearance of an extrinsic dichroic band in the absorption region of the reduced nicotinamide ring. The rotational strength of this extrinsic band is influenced by conformational alterations of the protein structure brought about by the binding of allosteric ligands. Titrations of protein sites with NADPH and threonine were performed by measuring changes in circular dichroic bands. The circular dichroic spectra in the far ultraviolet and the optical rotatory dispersion spectra in the visible and near ultraviolet were used to estimate the fraction of helical residues in the native protein and in its complexes. The methods are in fair to poor agreement. Taken together, the various methods suggest the possible presence in the protein of other types of peptide structure than the α helix and random coil.

The mechanisms of cooperative conformational changes in proteins is a subject of continuing interest. Circular dichroic and optical rotatory dispersion spectroscopy are powerful tools capable of providing detailed information about the nature of the conformational changes which may occur. Structural and quantum mechanical interpretations of such spectra have been discussed in several recent review articles (Beychok, 1966; Ulmer and Vallee, 1965; Blout *et al.*, 1967; Eyring *et al.*, 1968; Schellman, 1968).

The present paper reports the results of an application of these techniques in an investigation of the binding equilibria and denaturation of aspartokinase I–homoserine dehydrogenase I of *Escherichia coli* K 12. This bifunctional enzyme has recently been the subject of a number of physical-chemical studies, primarily in the laboratory of G. N. Cohen and his collaborators. These workers have demonstrated the cooperative nature of threonine binding and resulting inhibition of the two enzymatic activities (Patte *et al.*, 1966). Strong evidence was presented that the two activities are associated on a single protein molecule (Patte *et al.*, 1966). The molecular weight, amino acid composition (Truffa-Bachi *et al.*, 1968), and subunit structure (Truffa-Bachi *et al.*, 1969) have been determined. Ligand binding has been investigated by a variety of techniques, including equilibrium dialysis, and ultraviolet absorption and fluorescence spectrophotometry (Janin *et al.*, 1969). These studies showed that at least one tryptophan residue is involved in the binding of NADPH. The thermodynamics of the conformational change between active and inhibited enzyme forms has been investigated (Janin and Cohen, 1969), and the kinetics has been studied by both stopped-flow (Barber and Bright, 1968; Janin and Iwatsubo, 1969) and temperature-jump techniques (Janin and Iwatsubo, 1969).

The fundamental studies of Cohen and his collaborators demonstrated that changes in the protein absorption and fluorescence spectra occur when allosteric ligands are bound (Janin *et al.*, 1969; Janin and Cohen, 1969). According to Janin and Cohen (1969), the spectroscopic effects were consistent with the two-state model of Monod *et al.* (1965); the allosteric ligands, aspartate, and potassium ion, shift a conformational equilibrium in favor of a catalytically active R form, whereas threonine binds preferentially to an inactive T form.

In the present paper, we report (1) the fraction of helical peptide bonds in the protein and the effect of ligand binding on helical content; (2) the behavior of the protein under denaturing conditions; and (3) the effect of ligand binding on intrinsic and extrinsic circular dichroic bands.

* From the Departments of Chemistry, and Bacteriology and Immunology, University of California, Berkeley, California 94720. *Received February 10, 1970.* This investigation was supported by Grant No. 5-RO1-AM13164-01 from the National Institute of Arthritis and Metabolic Diseases, U. S. Public Health Service.

† Present address: Service de Physiologie Microbienne, Institut Pasteur, Paris 15è, France.

Experimental Section

Materials. L- and D,L-threonine and aspartic acids were purest grade amino acids obtained from either Sigma or Calbiochem. The aspartic acids were neutralized with KOH or NaOH. Dithioerythritol and NADPH were obtained from Sigma. Tetramethylammonium chloride and guanidinium chloride were Eastman products. The latter was recrystallized from absolute ethanol before use. Buffers and salts were reagent grade quality.

E. coli K 12, strain Tir 8 (Szentirmai *et al.*, 1968), was grown on a minimal medium containing 1% glucose as the carbon source. The cells were harvested in the late log phase and stored at −15°. The homogeneous protein was isolated from bacterial extracts as described by Truffa-Bachi *et al.* (1968). The enzyme was estimated to be between 95 and 98% pure by polyacrylamide gel electrophoresis. After purification, the enzyme preparation, crystallized in 40% $(NH_4)_2SO_4$ containing 2×10^{-3} M L-threonine, was maintained at room temperature. The activity was measured as described by Truffa-Bachi *et al.* (1968).

Buffer K contained 20 mM potassium phosphate, 2 mM magnesium-EDTA, and 0.15 M KCl, pH 7.20. Buffer T consisted of 20 mM sodium phosphate, 2 mM magnesium-EDTA, and 0.15 M tetramethylammonium chloride, pH 7.20. The protein resuspended from ammonium sulfate precipitate was equilibrated with buffer by passing it down a short column of Sephadex G-25. Protein concentrations were determined using an extinction coefficient at 278 nm of 0.46 cm^2/mg (Janin *et al.*, 1969).

Methods. Circular dichroic and optical rotatory dispersion spectra were measured on a Cary 60 spectropolarimeter connected to a PDP-8/S computer (Tomlinson, 1968). The program averages the spectropolarimeter pen reading over a specified wavelength region. This procedure is repeated at specified wavelength intervals within a specified range. An experimental base line is subtracted, and the teletype prints the difference between the spectrum and base line, multiplied by an arbitrary constant. A check on the computer output was run with lactate dehydrogenase, and gave good agreement with published optical rotatory dispersion spectra (Jaenicke, 1964).

The cell compartment was thermostated at 25 ± 0.5°. Cells with a light path of 10.0 and 1.0 mm were used. Measurements of pH were performed on a Radiometer type 28 pH meter. For spectral measurements, ligand concentrations were used which were at least 6 times greater than known dissociation constants (Janin and Cohen, 1969). Unless otherwise indicated in the figures, molecular ellipticities are based on decimoles of protein molecules. The molecular weight of the protein is 360,000 daltons (Truffa-Bachi *et al.*, 1968).

Mean residue rotations, corrected for solvent refractive index, were calculated from the measured values using eq 1, where *mrw* is the mean residue weight (108) (Truffa-Bachi *et al.*, 1968), and *n* the solvent refractive index. Refractive

$$[m']_\lambda = \frac{3\ mrw}{100(n^2 + 2)}[\alpha]_\lambda \qquad (1)$$

index corrections for [*m*] in water and in 6 M guanidinium chloride were measured in the visible region using white light, or, at shorter wavelengths, were obtained from tables (Fasman, 1963) or were calculated from a corrected form of the Sellmeier equation (Tanford *et al.*, 1967). The dispersion of the refractive index was only 3% over the wavelength region in which optical rotatory dispersion measurements were made (600–300 nm).

Rotational strengths, corrected for solvent refractive index, were calculated by graphical integration of circular dichroic curves using

$$R_k' = 6.961 \times 10^{-43}\left(\frac{3}{n^2 + 2}\right)\int_0^\infty \frac{[\theta_k]}{\lambda}d\lambda \qquad (2)$$

or, by assuming Gaussian bands, using

$$R_k' = 1.233 \times 10^{-42}\left(\frac{3}{n^2 + 2}\right)\frac{[\theta_k{}^\circ]\Delta_k{}^\circ}{\lambda_k{}^\circ} \qquad (3)$$

where $[\theta_k]$ is the molecular ellipticity in deg-cm^2/dmole, $[\theta_k{}^\circ]$ the maximum value of $[\theta_k]$ occurring at $\lambda_k{}^\circ$, and $\Delta_k{}^\circ$ equal to one-half the bandwidth at a bandheight of 0.368 $[\theta_k{}^\circ]$ (Moscowitz, 1960). The two methods gave identical rotational strengths within experimental error.

The results of the rotatory dispersion measurements were analyzed according to the Moffitt–Yang (1956) equation and the modified two-term Drude equation of Shechter and Blout (1964a). Helical content was calculated by three methods: (1) the depth of the circular dichroic trough at 222 nm, using mean residue ellipticities −38,000 for pure, right-handed α helix and +3000 for random coil (Javaherian and Beychok, 1968); (2) the slope of the Moffitt–Yang (1956) plot, using $\lambda_0 = 212$ nm, where b_0 for pure helix is −700 and for random coil +100 (Shechter *et al.*, 1964); (3) the equations of Shechter and Blout (1964a), using for pure helix $A_{(\alpha,\rho)193} = +2900$, $A_{(\alpha,\rho)225} = -2050$, and for random coil $A_{(\alpha,\rho)193} = -750$, $A_{(\alpha,\rho)225} = -60$. Other calculations, using the $A_{(\alpha,\rho)}$ values obtained for organic solvents (Shechter and Blout, 1964b), were also employed.

Results

Except for the measurements in guanidinium chloride, the circular dichroic and optical rotatory dispersion spectra reported in this paper were obtained at a constant ionic strength of 0.2 M. The composition of the buffer is, however, of considerable importance. As shown by Patte *et al.* (1963) and by Wampler and Westhead (1968), both activities of aspartokinase I–homoserine dehydrogenase I are activated by potassium ion. At high concentrations, sodium ion is reported to inhibit the dehydrogenase activity (Patte *et al.*, 1963), and to cause a very slight activation of the kinase (Wampler and Westhead, 1968). There was no measurable kinase activity either in the absence of any added salts or in the presence of tetramethylammonium ion (Wampler and Westhead, 1968).

Recently, Janin and Cohen (1969) have investigated the binding of monovalent cations to the enzyme. These authors demonstrated that potassium binding, but not sodium binding, shows positive cooperative effects, and have interpreted their results in terms of the allosteric model of Monod *et al.* (1965), potassium ion being classified as an allosteric effector.

Because potassium ion is unique in its ability to activate

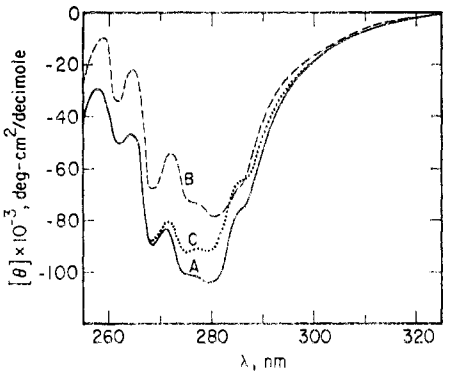

FIGURE 1: Near ultraviolet circular dichroic spectra of aspartokinase I–homoserine dehydrogenase I in buffer K: free enzyme (A); enzyme + 2 mM L-threonine (B); enzyme + 20 mM potassium L-aspartate (C).

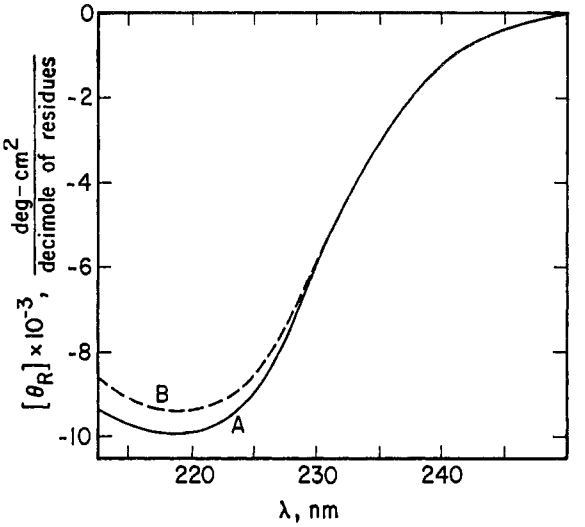

FIGURE 3: Far ultraviolet circular dichroic spectra of aspartokinase I–homoserine dehydrogenase I. Free enzyme in buffer K or enzyme + 16 mM potassium D,L-aspartate in buffer K (A); enzyme + 2 mM D,L-threonine in buffer K or T, free enzyme in buffer T, or enzyme + 16 mM sodium D,L-aspartate in buffer T (B).

the enzyme and to bind in a cooperative manner, it was of interest to measure the circular dichroic spectrum of the enzyme in the presence and absence of potassium ion, but at a constant ionic strength. For purposes of comparison, it was desirable to control the ionic strength with a nonactivating cation. Experiments were therefore carried out in the presence of tetramethylammonium chloride.

In addition, experiments which are not reported in detail were performed without added salt in 20 mM sodium phosphate buffer containing 2 mM magnesium-EDTA, pH 7.20. The effects of ligand binding on the spectra obtained in this buffer were very similar to results found in buffer T.

Circular Dichroic Spectra in Buffer K. The near ultraviolet circular dichroic spectrum of aspartokinase I–homoserine dehydrogenase I in buffer K is shown in Figure 1, curve A. The spectrum is seen to be a complex overlap of a number of negative bands. The bands probably arise from aromatic transitions, although at shorter wavelengths there may be a contribution from disulfide bonds, there being one cystine in each of the six subunits (Truffa-Bachi *et al.*, 1969). Addition of the optically inactive disulfide reducing agent, dithioerythritol, has no effect on the circular dichroic spectrum of the native enzyme.

If the protein is denatured, the circular dichroic spectrum is altered considerably. In 6 M guanidinium chloride, the spectrum changes to that shown in Figure 2, curve A. In this figure, the spectrum of the native enzyme (curve B) is included for comparison. This result demonstrates the importance of conformation in determining the appearance of the circular dichroic spectrum of this protein. The spectra in guanidinium chloride reported here and in the following discussions were taken 2–4 hr after concentrated guanidinium chloride had been added to the protein solution. The same spectra were scanned 13 hr later with no observable difference from earlier measurements.

It is known that in 6 M guanidinium chloride the protein is dissociated into its constituent subunits (Truffa-Bachi *et al.*, 1969). Furthermore, from the work of Tanford and his associates (Tanford, 1968), proteins in 6 M guanidinium chloride may be expected to behave as random coils, whether or not their disulfide bonds are reduced. We were therefore surprised to discover that addition of dithioerythritol to the denatured protein led to a change in the near ultraviolet circular dichroic spectrum at wavelengths considerably removed from the usual region of cystine dichroism [250–270 nm (Beychok, 1966)] (Figure 2, curve C). Particularly noteworthy is the disappearance of the positive band at 295 nm. This band is most probably due to a tryptophan, suggesting that the tryptophan concerned is located somewhere near the disulfide bond, and is either interacting directly with it or with other amino acids whose relative motion is not random because of the disulfide linkage.

The deep ultraviolet circular dichroic spectrum of the native protein in buffer K is shown in Figure 3, curve A. The broad negative band centered at about 218 nm suggests the possible presence of both α-helical and antiparallel β-chain structures (Timasheff and Gorbunoff, 1967). The $n-\pi^*$ trough is usually

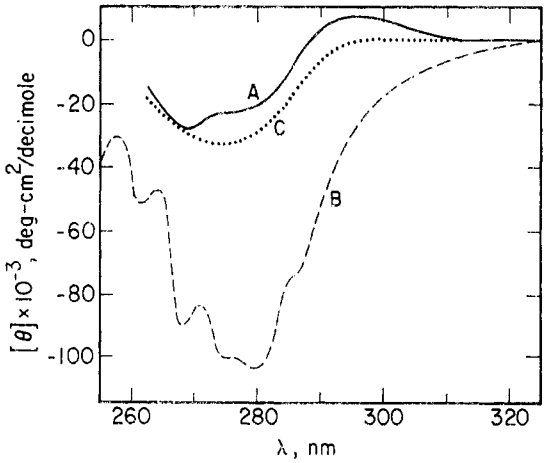

FIGURE 2: Near ultraviolet circular dichroic spectra of aspartokinase I–homoserine dehydrogenase I in 6 M guanidinium chloride (A) and in 6 M guanidinium chloride + 0.2 mM dithioerythritol (C). The spectrum of the native enzyme in buffer K is included for comparison (B).

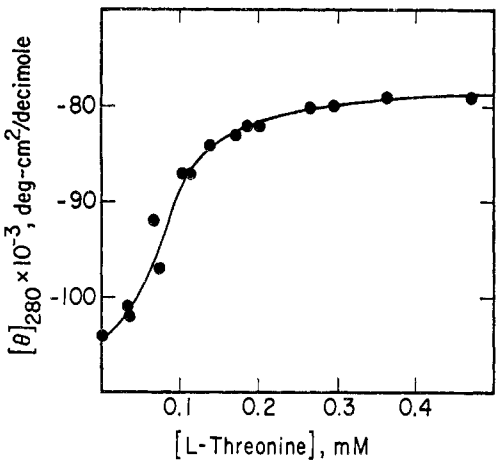

FIGURE 4: Circular dichroic titration of aspartokinase I–homoserine dehydrogenase I with L-threonine. The concentration of enzyme was 5 μM.

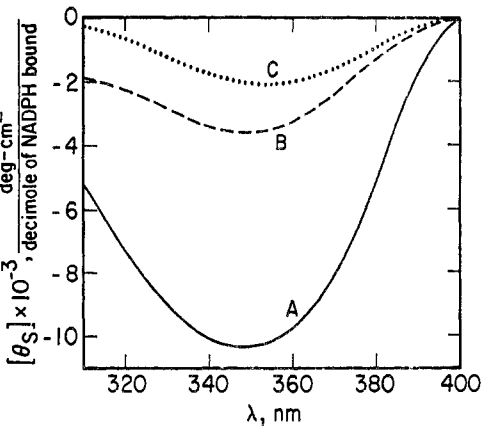

FIGURE 5: Near ultraviolet circular dichroic spectra of NADPH complexes of aspartokinase I–homoserine dehydrogenase I. Complex in buffer K (A); complex in buffer K + 2 mM L-threonine (B); complex in buffer T (C). In the absence of enzyme, NADPH is not measurably dichroic at 25° in this wavelength region.

found at 222 nm in right-handed α-helical polypeptides, whereas the band due to β structure is generally shifted to slightly shorter wavelengths (Timasheff and Gorbunoff, 1967).

In the presence of 0.15 M KCl, threonine binding shows positive cooperativity (Janin *et al.*, 1969). The effect of threonine binding on the circular dichroic spectrum is shown in Figure 1, curve B, and in Figure 3, curve B. Threonine binding leads to decreases in the magnitudes of the ellipticity bands throughout the wavelength region.

The change in molecular ellipticity at 280 nm was used to titrate the protein with threonine. The titration curve, shown in Figure 4, is strongly sigmoidal. The concentration of threonine required for a fractional ellipticity change of one-half at 280 nm is the same concentration previously found by equilibrium dialysis to be necessary for half-saturation of the sites (Janin *et al.*, 1969), indicating that the circular dichroic spectral change at this wavelength is essentially measuring the saturation function.

In the absence of threonine and in buffer K, aspartate binding is apparently not cooperative (Janin and Cohen, 1969). The effect of aspartate on the circular dichroic spectrum is markedly different from that of threonine. As shown in Figure 1, curve C, aspartate binding leads to only a small change at the long-wavelength side of the near ultraviolet circular dichroic spectrum. This may be interpreted either as a specific interaction of aspartate with protein chromophores or as a local conformational change resulting from aspartate binding.

At shorter wavelengths, aspartate binding has no effect on the circular dichroic spectrum. The deep ultraviolet circular dichroic spectrum of the enzyme in buffer K in the presence of 16 mM potassium D,L-aspartate is identical with that of Figure 3, curve A.

The binding of NADPH is known to be essentially unaffected by the presence or absence of threonine in buffer K and is not cooperative (Janin *et al.*, 1969). In the near ultraviolet, NADPH binding results in the appearance of an extrinsic dichroic band at 348 nm (Figure 5, curve A). Similar dichroic bands or extrinsic Cotton effects have already been observed with other dehydrogenases, including alcohol dehydrogenase (Ulmer and Vallee, 1965; Rosenberg *et al.*, 1965), glyceraldehyde 3-phosphate dehydrogenase (De Vijlder and Harmsen, 1969), and glutamate and lactate dehydrogenase (H. Heck, unpublished results), and may be expected to be a general phenomenon with all such proteins. The rotational strength of the induced band, per NADPH bound, is listed in Table I. The extrinsic dichroic bands observed in other dehydrogenases are of comparable rotational strengths, although there exist differences in sign.

The rotational strength of the extrinsic dichroic band of NADPH depends on the conformational state of the protein. As shown in Figure 5, curve B, and in Table I, binding of threonine results in a pronounced decrease of the rotational strength. A reduction of the rotational strength from that observed in buffer K is also found if the protein is dissolved in buffer T (see later).

The change in molecular ellipticity at 340 nm was used to titrate the protein with NADPH (Figure 6) to 15-fold higher ligand concentrations than were possible with fluorometric methods (Janin *et al.*, 1969). The experiments failed to reveal the existence of any weak-binding NADPH sites. The number of sites found, 3 ± 0.3 per hexamer, was in agreement with the fluorometric determination (Janin *et al.*, 1969).

The binding of NADPH has no measurable effect on the deep ultraviolet circular dichroic spectrum of the protein, either in the presence or absence of threonine. Thus, if an

TABLE I: Rotational Strengths of Extrinsic Dichroic Bands per NADPH Bound.[a]

State of Protein	R_k' (cgs)
Dissolved in buffer K	-10.1×10^{-40}
Dissolved in buffer K + 2×10^{-3} M L-threonine	-3.0×10^{-40}
Dissolved in buffer T	-1.6×10^{-40}

[a] Conditions described in the text.

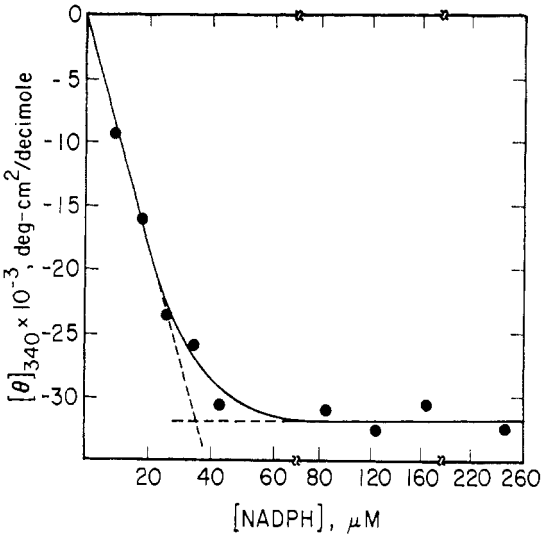

FIGURE 6: Circular dichroic titration of aspartokinase I–homoserine dehydrogenase I with NADPH. The concentration of enzyme was 12.9 μM. The extrapolated lines intersect at 36 μM.

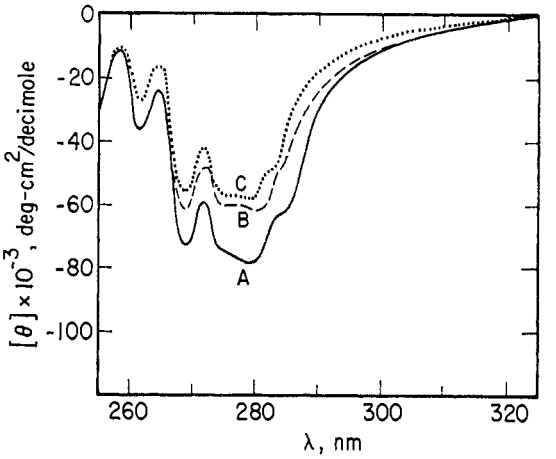

FIGURE 7: Near ultraviolet circular dichroic spectra of aspartokinase I-homoserine dehydrogenase I in buffer T. Free enzyme (A); enzyme + 2 mM L-threonine (B); enzyme + 20 mM sodium L-aspartate (C).

alteration of the peptide backbone occurs, it is not measurable by these techniques.

Circular Dichroic Spectra in Buffer T. The near ultraviolet circular dichroic spectrum of the native enzyme in buffer T is shown in Figure 7, curve A. This spectrum is seen to be nearly identical with that of the enzyme–threonine complex in buffer K (Figure 1, curve B). Furthermore, the deep ultraviolet circular dichroic spectrum of the enzyme in 0.15 M tetramethylammonium chloride is superimposable on that of Figure 3, curve B. These results indicate the probable conformational similarity of the free enzyme in buffer T to the enzyme–threonine complex in buffer K, a result expected from an earlier hypothesis (Janin and Cohen, 1969).

If threonine is added to the enzyme in the presence of 0.15 M tetramethylammonium chloride, there is a small alteration of the circular dichroic spectrum in the near ultraviolet (Figure 7, curve B). The effect of threonine binding on the circular dichroic spectrum of the enzyme in buffer T is similar to the effect of aspartate binding on the circular dichroic spectrum of the enzyme in buffer K (Figure 1, curve C). In both cases, a minor decrease occurs in the magnitudes of the circular dichroic bands at wavelengths greater than about 267 nm. In neither case is there a change in the circular dichroic spectrum below this wavelength.

Aspartate binding in buffer T has a quite different effect from that in buffer K. In the near ultraviolet, the binding of aspartate results in a decrease in band intensities at wavelengths above 257 nm (Figure 7, curve C). The decrease in the band centered at 262 nm is particularly noticeable. No similar decrease occurs when aspartate binds to the potassium form of the enzyme (Figure 1, curve C). Furthermore, in the deep ultraviolet, the spectrum of the enzyme–aspartate complex in buffer T is identical with those of the enzyme alone and of the enzyme–threonine complex in the same buffer; thus, aspartate binding does not lead to a measurable structural alteration in the peptide backbone under these conditions.

The binding of NADPH to the enzyme in buffer T gives rise to an extrinsic dichroic band (Figure 5, curve C) as was shown to be the case in buffer K. The rotational strength of this band, given in Table I, is, however, considerably less than that of the enzyme–NADPH complex in buffer K, but is close to that of the enzyme–NADPH–threonine complex in the latter buffer. These results, together with the protein circular dichroic spectra noted earlier, provide strong evidence that the conformation of the protein in buffer T is essentially the same as that of the enzyme–threonine complex in buffer K, in agreement with the hypothesis of Janin and Cohen (1969).

Optical Rotatory Dispersion Spectra. Optical rotatory dispersion may provide information regarding the presence of other types of structure than the α helix and random coil in proteins. The method used is that of Shechter and Blout (1964a), and employs a modified two-term Drude equation from which two estimates of helical content can be made. If the estimates are self-consistent and in good agreement with other data, it may be concluded that the gross structure of the protein involves a combination of α-helical and random coil segments. Inconsistency in the estimates of helical content by the various methods may indicate that other conformations are present, although this interpretation does not yet have theoretical justification.

The results of calculations of helical content by several techniques are shown in Table II. It is seen that the results are in fair to poor agreement. Of particular interest is the inconsistency in helical contents calculated from the modified two-term Drude equation. The $A_{(\alpha,\rho)}$ values which we obtain do not satisfy the Shechter–Blout equations for proteins dissolved in aqueous solution (1964a). The discrepancy is reminiscent of results obtained by the latter authors for β-lactoglobulin and pepsinogen (Shechter and Blout, 1964a). In their cases as in ours, the helical content calculated from $A_{(\alpha,\rho)193}$ exceeded that obtained from $A_{(\alpha\ \rho)225}$ by approximately a factor of two.

Moffitt–Yang (1956) plots of optical rotatory dispersion spectra obtained with the native protein in buffer K and with the denatured protein in 6 M guanidinium chloride in the presence and absence of dithioerythritol are shown in Figure 8. This figure demonstrates the marked effect of denaturation

TABLE II: Fractions of α-Helical Residues in Free Enzyme and Enzyme Complexes Calculated by Various Methods.[a]

Protein or Protein Complex	% Helix Calculated from			
	Circular Dichroism$_{222}$	Moffitt–Yang Equation	Modified 2-Term Drude Equation	
			H_{193}	H_{225}
Free enzyme, buffer K	31	26	30	17
Free enzyme, buffer T	30			
Enzyme–threonine	30	26	30	17
Enzyme–aspartate, buffer K	31			
Enzyme–aspartate, buffer T	30			
Enzyme–NADPH, buffer K	31	26	31	18
Enzyme–NADPH–threonine	30	26	30	17

[a] Methods described in the text.

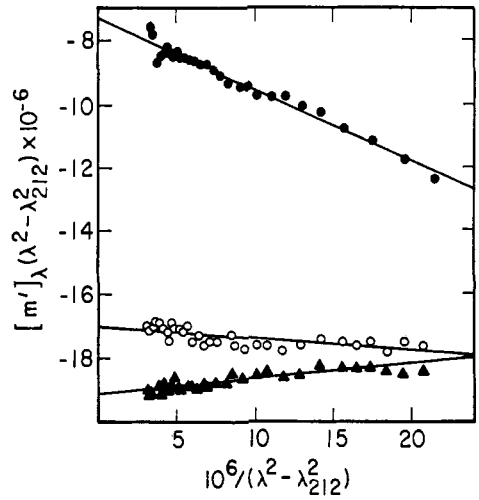

FIGURE 8: Moffitt–Yang plots of optical rotatory dispersion spectra of aspartokinase I–homoserine dehydrogenase I in buffer K (●), in 6 M guanidinium chloride (○), and in 6 M guanidinium chloride + 0.2 mM dithioerythritol (▲).

on the optical rotatory dispersion spectrum of the enzyme. The figure also shows that when dithioerythritol is added to the protein dissolved in 6 M guanidinium chloride, the spectrum undergoes a further change resulting in a Moffitt–Yang (1956) plot with positive slope. Thus, in agreement with the circular dichroic data of Figure 2, these results indicate that the protein is incompletely unfolded in 6 M guanidinium chloride unless disulfide bonds are reduced.

Discussion

The presence of a circular dichroic band signifies the existence of optical activity in the vicinity of an absorption band. This activity can arise both from intrinsic and from induced dissymmetry. In general, induced dichroism due to environmental factors in proteins is of much greater magnitude than the intrinsic dichroism of amino acid residues (Beychok, 1966). This is seen to be the case in aspartokinase I–homoserine dehydrogenase I, where, in the presence of 6 M guanidinium chloride, the structure and intensities of the circular dichroic spectra are greatly diminished.

The depth of the circular dichroic trough at 222 nm has been used to measure the fraction of peptide groups present in α-helical conformations (Javaherian and Beychok, 1968). Such a calculation is useful if evidence is available that only two types of structure are present in the protein: α helix and random coil, the latter term signifying merely the absence of a regularly repeated conformation of peptide bonds. Although such data are often lacking, the evidence for the presence of other types of peptide structure might be obtained from optical rotatory dispersion spectra. The work of Shechter and Blout (1964a) suggests that the presence of other types of structure is indicated if the fractions of helix calculated from the parameters $A_{(\alpha,\rho)193}$ and $A_{(\alpha,\rho)225}$ do not agree. This interpretation, however, does not yet have a sound theoretical basis. Disagreement between the helical contents calculated from the Shechter–Blout equations (1964a) therefore cannot be taken as proof that other types of structure are present.

The data of Table II show that the fractions of α helix calculated from $A_{(\alpha,\rho)193}$ and $A_{(\alpha,\rho)225}$ differ by nearly a factor of two. However, if the empirical equations for proteins dissolved in organic solvents are used (Shechter and Blout, 1964b), which might be applicable, for example, if large portions of the helical regions were not in contact with water (Harrison and Blout, 1965), the discrepancy between the two values for helical content decreases to approximately 25%. The limitations of theory do not permit an unequivocal interpretation of such discrepancies at this time. Clearly, the effect of local environment can be of great significance.

If the disagreements among the helical contents for aspartokinase I–homoserine dehydrogenase I calculated by various methods mean that other types of structure are present, the deep ultraviolet circular dichroic spectra suggest that at least some of this structure is in the form of the antiparallel pleated sheet. This hypothesis is supported by the observation that the deep ultraviolet circular dichroic trough is maximally intense at about 218 nm in agreement with that of the antiparallel β-chain conformation, whereas in model α-helical polypeptides the intensity maximum occurs at 222 nm (Timasheff and Gorbunoff, 1967).

It would, of course, be desirable to be able to interpret the contributions of various peptide structures to the optical rotatory dispersion spectra in a quantitative fashion, and attempts have been made in this direction (Greenfield *et al.*, 1967; Magar, 1968; Straus *et al.*, 1969). As yet, these attempts, which are based on the optical rotatory dispersion spectra of model polypeptides, have not been successful, as judged from X-ray crystallographic data.

From the deep ultraviolet threonine binding spectrum in buffer K (Figure 3), it is evident that changes occur in the far ultraviolet when threonine is bound to the enzyme. As noted above, however, the deep ultraviolet circular dichroic spectra

TABLE III: Moffitt–Yang Parameters and Mean Residue Rotations at 589 nm of Aspartokinase I–Homoserine Dehydrogenase I.

State of Protein	b_0	a_0	$[m']_{589}$ (deg cm²/dmole)
Native, buffer K	−110	−164	−27
Denatured in 6 M Gu·HCl,[a] –S–S– bonds intact	−17	−379	−57
Denatured in 6 M Gu·HCl, –S–S– bonds reduced	+24	−426	−63

[a] Gu·HCl is guanidinium hydrochloride.

cannot be correlated directly with α-helical content. Thus, the available data are insufficient to show whether this spectral change is due to an α-helix–random coil transformation in the main polypeptide chain. From the magnitude of the effect, however, one may judge that if such a change occurs, it involves a very small fraction of the amino acids.

The far ultraviolet circular dichroic spectra do indicate that potassium ion binding exerts a special effect either on the conformation of the peptide backbone or on the local anisotropy of certain peptide bonds. The depth of the trough is about 10% larger in buffer K than in buffer T (Figure 3). Binding of aspartate, on the other hand, does not exert a similar influence as potassium and aspartate binding in either buffer have no measurable effect on the depth of the far ultraviolet circular dichroic trough. In addition, potassium and aspartate binding have opposite effects on the near ultraviolet circular dichroic spectrum of the threonine form. Potassium binding leads to increases (Figure 1, curves A and B) while aspartate binding leads to decreases (Figure 7, curves B and C) in the intensities of near ultraviolet circular dichroic bands. Thus, both near and far ultraviolet circular dichroic spectra indicate differences either in conformation or local anisotropy between the potassium and aspartate complexes.

The measured near ultraviolet circular dichroic spectra of the various conformational states were obtained in the presence of allosteric ligands, and thus are perturbed by ligand binding. The magnitude of these perturbations can be estimated by observing the effects of threonine binding on the protein spectrum in buffer T (Figure 7, curves A and B) and of aspartate binding on the protein spectrum in buffer K (Figure 1, curves A and C). It is seen that these ligands cause small changes which can only be observed at wavelengths greater than about 267 nm. These perturbations may be due either to specific interactions of the ligands with protein chromophores or to local conformational rearrangements.

On the other hand, in those cases in which cooperativity of binding has been demonstrated, *i.e.*, threonine binding in buffer K and aspartate binding in the absence of potassium ion (Janin and Cohen, 1969), it is seen that the binding of these ligands causes significant perturbations of the circular dichroic spectra at wavelengths below 267 nm (Figure 1, curves A and B, and Figure 7, curves A and C). These shorter wavelength effects appear, therefore, to be in some manner related to the cooperative conformational rearrangements. From the location of these spectral perturbations, one is led to suspect the probable involvement of aromatic residues other than tryptophan, or possibly the disulfide bridges (Beychok, 1966). Since, however, the spectra were not perturbed by the addition of dithioerythritol, an exact identification of the groups giving rise to the ellipticity bands is not at present possible.

The extrinsic dichroic band centered at about 348 nm due to NADPH binding is of considerable interest. As shown in Figure 5 and in Table I, the rotational strength of this band is reduced both by threonine binding in buffer K and by removal of potassium ion. These observations call to mind the work of Stryer and Blout (1961) showing the effect of an α-helix–random coil transformation in a synthetic polypeptide chain on the optical activity of a bound dye molecule, although the underlying physical causes for the effects depicted in Figure 5 are certainly different in details from the examples of Stryer and Blout.

With regard to the latter point, it has been demonstrated by fluorescence techniques that the efficiency of energy transfer from an electronically excited tryptophan residue to the nicotinamide ring in buffer K is lowered by binding of threonine (Janin *et al.*, 1969). Thus, the fluorescence results suggest that, to a first approximation, the extrinsic dichroic band of NADPH may be interpreted in terms of dipolar interactions of the respective transition moments of nicotinamide and a nearby tryptophan. Calculations designed to test this hypothesis are in progress.

The effects of 6 M guanidinium chloride on the circular dichroic and optical rotatory dispersion spectra are of importance with regard to the effectiveness of guanidinium chloride as a denaturing agent. Tanford (1968) showed that a number of proteins in concentrated guanidinium chloride solution are randomly coiled when their disulfide bonds are intact as well as when they are broken. The data presented in this paper show that the present protein does not behave in this manner. As shown in Figure 2, the near ultraviolet circular dichroic spectra give evidence for some degree of structure remaining unless the disulfide bonds are reduced.

This conclusion is further substantiated by the optical rotatory dispersion measurements. In Table III are listed b_0 and a_0 values for the native and denatured proteins, obtained from the Moffitt–Yang (1956) plots of Figure 8. It is seen that denaturation of the protein and subsequent reduction of disulfide bonds by dithioerythritol both lead to positive shifts in the b_0 value from that of the native enzyme and to increasingly negative values for a_0. The last value for b_0 in Table III may be compared with the most recent estimate for b_0 in random polypeptide chains, which is +100 (Shechter *et al.*, 1964). The changes in a_0 are likewise in the expected direction for successively greater unfolding and exposure of peptide groups to the solvent (Blout *et al.*, 1967). These data therefore indicate that reduction of disulfide bonds leads to a significant further unfolding of the protein in 6 M guanidinium chloride to a state in which helical structures are virtually absent.

Further information regarding the state of the protein in 6 M guanidinium chloride is shown in the last column of Table III, in which are reported the values of mean residue rotations of the protein, corrected for solvent refractive index,

under various conditions at 589 nm. Notable is the negative shift in $[m']_{589}$ when dithioerythritol is added to the denatured enzyme. Such shifts have frequently been observed in urea, in which many proteins are incompletely unfolded (Tanford, 1968), but have heretofore never been observed in 6 M guanidinium chloride. In the latter solvent, in fact, the shifts have invariably been positive, with values ranging from +1 to +12 degrees (Tanford, 1968).

A positive shift would be expected if reduction of the disulfide bonds were the sole factor contributing to the rotation change at 589 nm, since $([m']_{589}^{CySH} - [m']_{589}^{Cys/2})$ is positive in sign (Tanford, 1968). On the other hand, destruction of ordered regions in an otherwise disordered chain would lead to an increase in levorotation. If the latter contribution to the rotation outweighed the former, a net negative shift would result. Thus, both the circular dichroic and optical rotatory dispersion evidence indicate that 6 M guanidinium chloride does not cause complete unfolding of this protein in the absence of disulfide reducing agents.

Two possible explanations might account for the fact that aspartokinase I–homoserine dehydrogenase I behaves differently from other proteins (Tanford, 1968) in being incompletely unfolded in 6 M guanidinium chloride unless disulfide bonds are reduced. First, if the protein contained an unusually large fraction of amino acids with nonpolar or aromatic side chains, these groups might interact hydrophobically and stabilize nonrandomly coiled configurations of the polypeptide chain. Second, even if the protein did not contain an unusually large fraction of amino acids with nonpolar or aromatic side chains, but the nonpolar side chains were concentrated near the disulfide bonds, the probability of their interacting would be greater when the bonds are intact than when they are broken. Either hypothesis could account for the disappearance of the 295-nm circular dichroic band observed in 6 M guanidinium chloride when dithioerythritol was added (Figure 2).

The first hypothesis is ruled out by the fact that the fraction of amino acids having nonpolar side chains in aspartokinase I–homoserine dehydrogenase I is about 50% (Truffa-Bachi *et al.*, 1968), which is comparable with the fraction of nonpolar side chains in several other proteins which appear to be completely unfolded in 6 M guanidinium chloride, both in the presence and absence of disulfide reducing agents (Tanford, 1968; Dayhoff and Eck, 1968). There is no evidence to support or disprove the second hypothesis at this time, since the amino acid sequence of the protein is not known. It is evident that further studies of the protein structure and denaturation mechanism are required if this hypothesis is to be adequately tested.

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