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Ultraviolet Rotatory Dispersion of Aspartic Aminotransferase*

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ABSTRACT: Rotatory dispersion measurements of the enzyme aspartic aminotransferase have been extended into the ultraviolet spectral region. All "native" forms of the enzyme, the pyridoxal, pyridoxamine, and oxime enzymes and the pyridoxal enzyme in the presence of excess substrates, have essentially the same ultraviolet dispersion curves which exhibit minima ($[\alpha] = -6600°$) at 231 mμ. The absolute rotation at the minimum is only slightly smaller for the apoenzyme ($[\alpha] = -5800°$). These results suggest the enzyme has a considerable amount of ordered structure. In 8 M urea the shape of the ultraviolet rotatory dispersion curve is considerably altered and the value of the rotation minimum is reduced to $[\alpha] = -4750°$. The visible portions of the rotatory dispersion curves (300–600 mμ) differ markedly for the various forms of the native enzyme owing to Cotton effects of the coenzyme. Although both the pyridoxal and pyridoxamine enzymes have marked Cotton effects, these Cotton effects disappear in the presence of high concentrations of the substrates. This suggests that rotatory dispersion measurements in the near ultraviolet and visible region of the spectrum may be useful as indicators of events occurring at the active site.

The rotatory dispersion properties of various forms of aspartic aminotransferase in the wavelength region 315–578 mμ have been previously reported (Fasella and Hammes, 1964a). Although pyridoxal and pyridoxamine phosphate are optically inactive, both the pyridoxal and pyridoxamine enzymes display Cotton effects centered about the wavelengths of the corresponding absorption maxima. On the other hand, the oxime enzyme, the apoenzyme, and the enzyme in urea appear to have plain dispersion curves. Similar results have been reported by Torchinsky and Koreneva (1963, 1964), who also found that other carbonyl reagents either abolished or inverted the coenzyme Cotton effect. The plain dispersion curves were analyzed according to the usual Moffit-Yang equation (Moffit and Yang, 1956). The resultant values of b_0 suggested that the apo- and denatured enzymes have very little ordered structure (e.g., α helices), while the oxime is highly ordered (Urnes and Doty, 1961); this indicates that the binding of coenzyme has an extraordinary effect on the protein structure. Unfortunately the theoretical basis for such an interpretation of the b_0 values is somewhat insecure; moreover b_0 values cannot be determined for other forms of the enzyme because of the Cotton effects occurring. In fact even in the case of the oxime enzyme a near-ultraviolet coenzyme Cotton effect might have given rise to an anomolous b_0 value.

An alternative measure of helical content or the ordered structure of proteins is the characteristic protein Cotton effect at about 232 mμ (Simmons *et al.*, 1961; Holzwarth *et al.*, 1962). Unfortunately a reliable theoretical analysis of this effect is also lacking, but in general this Cotton effect appears to be sensitive to changes in protein structure and should not be significantly influenced by the coenzyme Cotton effects occurring at longer wavelengths.

We present here the results of a study of the ultraviolet rotatory dispersion of various forms of aspartic aminotransferase. In addition the rotatory dispersion of the enzyme in the presence of excess substrates has been investigated.

Experimental

The preparation of materials was exactly as previously described (Fasella and Hammes, 1964a). Optical rotation was measured with a Cary 60 spectropolarimeter. All measurements were carried out at

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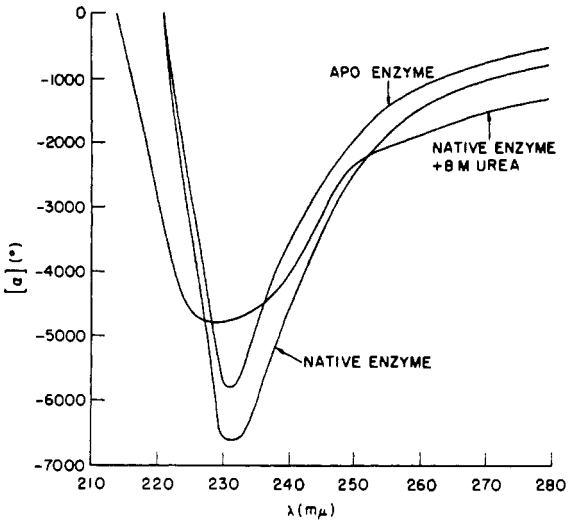

FIGURE 1: Ultraviolet optical rotation of aspartic aminotransferase. The term "native" enzyme includes aldimine enzyme, pyridoxamine enzyme, oxime enzyme, and aldimine enzyme in the presence of substrates. See the experimental section for details.

27° ± 1°. Measurements were made on at least two concentrations of every sample to ensure the absence of anomolous scattering effects. Protein concentrations were determined spectrophotometrically at 280 mμ and ranged from 0.1 to 10 mg/ml. Blanks were run on solutions identical to the sample of interest except that they did not contain enzyme. The optical rotatory power, $[\alpha]_\lambda$, was calculated from the usual equation

$$[\alpha]_\lambda = \frac{100\alpha}{lc}\lambda$$

where l is the cell length in decimeters, c is the concentration of optically active material in 100 ml, and α_λ is the observed rotation corrected for the solvent blank. The following forms of the enzyme were studied at both *p*H 5 (0.2 M sodium acetate buffer) and 8 (0.2 M potassium phosphate buffer): aldimine enzyme, pyridoxamine enzyme, apoenzyme, and oxime enzyme (10^{-3} M hydroxylamine). The aldimine enzyme in 8 M urea and in the presence of substrates, aspartate (5×10^{-2} M)-oxalacetate (2×10^{-3} M) or glutamate (5×10^{-2} M)-ketoglutarate (1×10^{-3} M), was studied at *p*H 8 (0.2 M potassium phosphate buffer). Racemic amino acids (Calbiochem) were used to eliminate optical rotation due to the substrates.

Results and Treatment of Data

In Figure 1, typical ultraviolet rotatory dispersion curves for the apoenzyme, the urea-denatured enzyme, and the "native" enzyme are given. The curve for the "native" enzyme is essentially identical in the wavelength region 220–280 mμ for the following forms of the enzyme: pyridoxal enzyme, pyridoxamine enzyme, and oxime enzyme at *p*H 5 and 8; and pyridoxal enzyme in the presence of excess substrates at *p*H 8.

It was found that the rotatory dispersion was quantitatively different for different enzyme preparations (±6% of the average values) so that the curves shown represent average results from several different experiments. Valid comparisons among the various forms of the enzyme can be made with only a single preparation. For example, the results for the pyridoxal and oxime enzyme were compared by first determining the curve characteristic of the pyridoxal enzyme and then adding a very small volume of concentrated hydroxylamine and redetermining the rotatory dispersion curve. The apoenzyme was reconstituted with concentrated pyridoxamine phosphate in a similar experiment. Average values of $[\alpha]_{231}$ for the various forms of the enzyme are presented in Table I. A value of $[\alpha]_{232}$ of approximately 7000° has recently been reported by Jirgensons (1964) for aspartic aminotransferase, but no mention was made concerning the form of the enzyme used for this study. The data obtained for the oxime enzyme, the apoenzyme, and the enzyme in the presence of excess substrates were of sufficient precision to indicate clearly the apparently plain curves are not adequately described by the Moffit-Yang treatment (Moffit and Yang, 1956) over an extended wavelength region (270–600 mμ). This point is illustrated by the results in Figure 2 which are plotted according to the Moffit equation. In order to cover the entire wavelength region, the ordinate and abscissa scales have been compressed; this somewhat deemphasizes the deviations of the points from the straight lines. However these deviations are real and reproducible. The inadequacy of this type of treatment is not so apparent when a more restricted wavelength region is considered. The deviations from the Moffit equation might be due to the presence of small coenzyme Cotton effects, or, in the case of the apoenzyme, to small amounts of coenzyme which may be present. In our opinion, an analysis of these rotatory dispersion curves in terms of the conventional a_o–b_o parameters is not very mean-

TABLE I: Ultraviolet Optical Rotation.

Enzyme[a]	$[\alpha]_{231}$ (°)
E_L, E_LH, E_M	−6600 ± 400
E_L, E_LH + NH_2OH	−6600 ± 400
Apoenzyme	−5800 ± 400
E_L + Gm-Kg	−6600 ± 400
E_L + As-Oa	−6600 ± 400
E_L in 8 M urea	−4750 ± 300

[a] Gm = glutamate, Kg = ketoglutarate, As = aspartate, Oa = oxalacetate, E_L = aldimine enzyme at *p*H 8, E_LH = aldimine enzyme at *p*H 5, E_M = pyridoxamine enzyme.

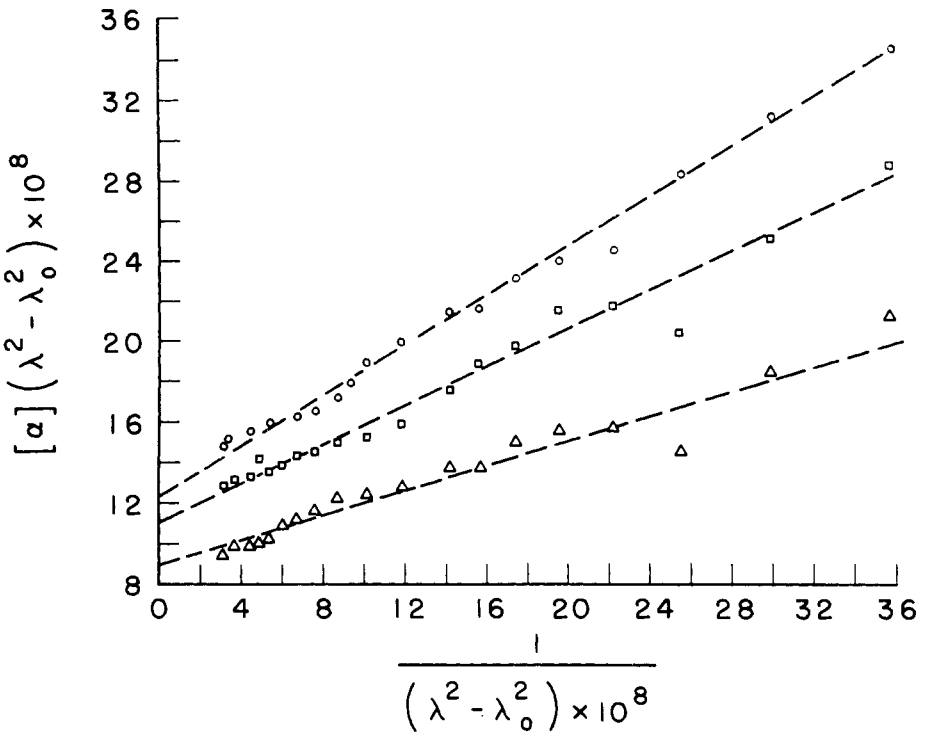

FIGURE 2: Plot of rotatory dispersion data according to Moffit and Yang (1956). Oxime enzyme (*p*H 8), ○; aldimine enzyme + glutamate-ketoglutarate (*p*H 8), □; and apoenzyme (*p*H 8), △.

ingful; nevertheless, because of the widespread usage of these parameters, the average a_0-b_0 values obtained are given in Table II. The values of the parameters for the oxime and apoenzyme differ somewhat from those previously reported. The primary reason for this is that in the previous work the wavelength region 315–500 mμ was weighted most heavily in analyzing the data; if this were done in the present case the apparent a_0-b_0 parameters would approach those previously reported.

The near ultraviolet and visible part of the rotatory dispersion curves of the enzyme in the presence of glutamate-ketoglutarate and aspartate-oxalacetate are given in Figure 3 together with those of some other forms of the enzyme for reference. The concentrations of substrates employed were such that only enzyme-substrate complexes should be present in significant amounts (Velick and Vavra, 1962; Hammes and Fasella, 1962). It should be noted that, although the dispersion curves of many forms of the enzyme are identical in the 220–280 mμ region, they all differ considerably in the near ultraviolet and visible owing to the Cotton effects associated with the coenzyme absorption bands.

Finally a comparison should be made between results obtained using a recording and a manual spectropolarimeter. The data are all in reasonable agreement above 325 mμ with the exception of the rotatory dispersion of the pyridoxamine enzyme. The pyridoxamine enzyme is extremely susceptible to denaturation, and partial denaturation probably occurred either during the preparation of the enzyme (the time for preparation has now been considerably reduced) or during measurements with the manual instrument. Thus the previously reported rotatory dispersion curve is more negative than the one shown in Figure 3, but the shape of the curve is unchanged. In all cases the data obtained with the recording instrument are to be preferred. At this point it should again be emphasized that the results obtained with different enzyme preparations showed some deviation so that considerable caution must be used in making detailed comparisons.

Discussion

One of the most striking features of the results obtained is the marked similarity of the ultraviolet rotatory dispersion for all active forms of the enzyme having a pyridoxal derivative bound. As shown in Table I, the minimum rotation values occurring at 231 mμ for these substances are essentially identical. Thus in the case of aspartate aminotransferase, the ultraviolet rotatory dispersion is virtually useless as an indicator for events occurring at the active site. Other enzyme systems also show this behavior, e.g., phosphoglucomutase (Sloane, 1964) and alcohol dehydrogenases (Kägi, 1964). If the rotation at 231 mμ is used as a criterion of ordered protein structure the apo- and native enzymes differ very little, contrary to what was concluded earlier on the basis of b_0 values calculated using data from a restricted wavelength region (Fasella and Hammes,

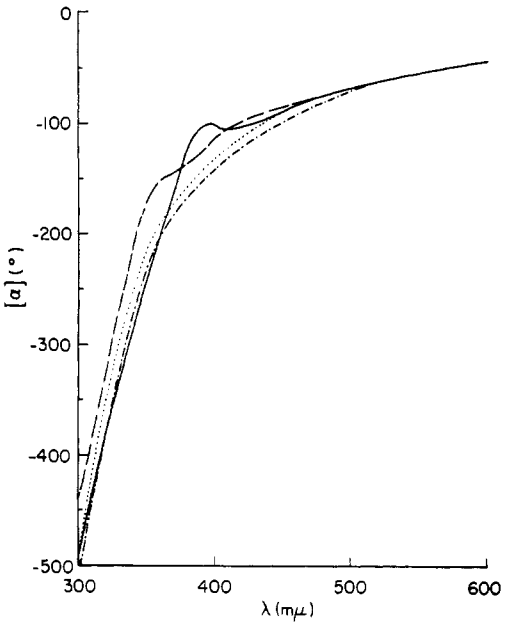

FIGURE 3: Rotatory dispersion of aspartic aminotransferase at *p*H 8. Aldimine enzyme, ———; pyridoximine enzyme, ---------; aldimine enzyme + aspartate-oxalacetate, ·–·–·–; and aldimine enzyme + glutamate-ketoglutarate, ·····. See experimental section for details.

TABLE II: Approximate Rotatory Dispersion Parameters.[a]

Enzyme Form	a_o (°)	b_o (°)
Apoenzyme	−180 (−215)	−130 (−90)
E_L-NH_2OH	−250 (−225)	−280 (−490)
E_L-Gm-Kg	−220	−220
E_L-As-Oa	−240	−240

[a] Symbols are as defined in Table I; values in parentheses are those previously reported (Fasella and Hammes, 1964a).

1964a), or what is indicated by the results in Table II. The visible part of the dispersion curve, on the other hand, is extremely sensitive to changes occurring at the active site of the enzyme. A similar situation has been found for the alcohol dehydrogenases (Kägi, 1964). Good examples of these phenomena are shown in Figure 3. Rotatory dispersion can be extremely valuable in interpreting the spectral properties of enzyme-substrate mixtures since free forms of the enzyme and enzyme-substrate complexes have similar absorption spectra, but only substrate free forms of the enzyme display clear-cut Cotton effects. We are presently attempting to exploit these rotatory dispersion properties for the determination of enzyme-substrate binding constants. The fact that the enzyme-substrate complexes do not display any apparent Cotton effects is probably owing to the lack of the strong interaction between the enzyme and the aldehyde and amino groups of the coenzyme which occurs in other forms of the enzyme (Turano *et al.*, 1961; Hughes *et al.*, 1962). The fact that the ultraviolet rotatory dispersion remains unchanged upon complex formation suggests that no gross changes in the ordered structure of the protein occur, although a reorientation of ordered structures is not precluded, nor are local conformational changes at the active sites.

The rotatory dispersion curve of the apoenzyme is markedly different from that for any other forms of the enzyme. The curve is much flatter and the absolute value of the minimum rotation in the ultraviolet range is somewhat lowered relative to the native enzyme, the difference being barely outside experimental error. The similarity of the ultraviolet rotatory dispersion curves for native and apoenzyme bears out the premise that for the native enzyme the rotation at 231 mμ is determined mainly by the protein structure, rather than by possible coenzyme Cotton effects. The optical rotation of the enzyme in urea is somewhat atypical: although the rotation from 300 to 600 mμ becomes more negative and the ultraviolet minimum becomes much more positive, the absolute value of the maximum rotation around 230 mμ is quite large, suggesting that a considerable amount of ordered structure in the enzyme may exist even after 48 hours in 8 M urea (Fasella and Hammes, 1964b); again this is an apparent contradiction with the conclusion one might reach on the basis of the b_o value (−60°) characteristic of the denatured enzyme. The fact that ultraviolet rotation values may give different estimates of ordered structure (helical content) than b_o values has also been pointed out by Jirgensons (1964) in a comparative study of the b_o and $[\alpha]_{233}$ values of a large number of proteins.

As these results suggest, an interpretation of rotatory dispersion measurements in terms of detailed protein structure is certainly not justified in view of the current status of experimental and theoretical work. On the other hand, the use of extrinsic Cotton effects as indicators of a change in environment at the active site has considerable potential for the elucidation of enzyme mechanisms (cf. Ulmer and Vallee, 1965).

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Location of Abnormal Tyrosines in Actin*

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ABSTRACT: Spectrophotometric titrations on G- and F-actin indicate that almost all the tyrosines are buried inside the molecule. Correlated with exposure of these buried tyrosines at high *p*H, an irreversible denaturation, or loss of polymerizability, took place. All the tyrosine residues were titrated at *p*H higher than 12 and were reabnormalized by lowering the *p*H, indicating that a refolding of the molecule occurred and masked some of the tyrosines. Guanidine HCl normalized the abnormal tyrosines, since a reversible titration curve with the normal *pK* of phenolic groups was obtained in 5 M guanidine HCl solution. In this solution, splitting of a monomer into two subunits occurs according to Adelstein and co-workers (R. S. Adelstein, J. E. Godfrey, and W. W. Kielley, *Biochem. Biophys. Res. Commun. 12*, 34 [1963]). When guanidine HCl was dialyzed out against water containing 30 mM KOH, the subunits did not recombine to form a monomer or some aggregate, as judged from the molecular weight (Archibald method) and the sedimentation constant obtained for this solution.

The effect of increasing the concentration of guanidine HCl added to actin solution at *p*H 10 demonstrated two steps in the normalization of the tyrosines, suggesting the existence of two kinds of abnormal groups. Another denaturing reagent, urea, could not normalize all the tyrosines, indicating that the action of urea is different from that of guanidine HCl. The results suggest that the molecule has a rigid core with deeply buried tyrosines.

Actin, one of the muscle proteins (Straub and Feuer, 1950), exists in a globular form (G-actin) of a molecular weight of about 6×10^4 in salt-free solutions (Mommaerts, 1952; Lewis *et al.*, 1963; Mihashi, 1964). On addition of neutral salts (e.g., KCl), G-actin polymerizes into a long fibrous molecule (F-actin) (Straub and Feuer, 1950). Electron-microscopic studies of F-actin by Huxley (1963) and by Hanson and Lowy (1963) show that G-actin molecules are arranged to form the double-stranded helical structure of F-actin, so that each G-actin molecule seems to interact with four neighbors by an attractive force of specific importance for the polymerization (Oosawa and Kasai, 1962; Asakura *et al.*, 1963). However, there are few reports on the structure of actin which elucidate the molecular mechanism of its polymerization (Higashi *et al.*, 1963).

Recently, Higashi and Oosawa have found a significant difference spectrum between G-actin and F-actin (S. Higashi and F. Oosawa, to be published), suggesting an increase in the content of α helix and/or β structure of the molecule associated with polymerization. Another important result has been obtained by Adelstein *et al.* (1963), who showed that a monomer of actin is composed of two subunits of similar, if not identical, mass; a value of 28,200 for the average molecular weight of these subunits has been determined by short-column sedimentation equilibrium in 5 M guanidine hydrochloride solution.

It is therefore of great importance to obtain further knowledge of the structure of the actin molecule, including side-chain interactions, by carrying out physicochemical measurements on actin solutions. With these results we expect to clarify the relationship between the structure and function (i.e., polymerizability) of actin.

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