THE ROTATIONAL RELAXATION TIME OF ASPARTATE AMINOTRANSFERASE

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The enzyme aspartate transaminase (L-Aspartate 2-OxOglutarate aminotransferase E.C. 2.6.1.1) contains two molecules of Pyridoxal-5 Phosphate per 110.000 Mw (1) and a quantitative determination of the terminal alanine residues showed that there are two moles of alanine per Mol of protein (2). Although these results appear to indicate that the enzyme is composed of two polypeptide chains, the molecular weight measurements, normally conducted at protein concentrations of the order of 5 mg per ml, did not reveal the presence of species of lower molecular weight (55.000) (3,4).

Since the two polypeptide chains may have a tendency to associate at high protein concentrations, it was thought of interest to investigate the molecular state of the enzyme aspartate transaminase at concentrations approaching those used in the enzymatic activity assays. To this end the method of polarization of fluorescence was used to determine the rotational relaxation time of the enzyme at a concentration of 0.04 mg/ml. Polarization of fluorescence measurements were conducted in a polarization photometer similar to that described by G. Weber (5). An analysis of the various sources of random and systematic errors shows that this device is capable of measuring the degree of polarization of fluorescence to an accuracy of 0.5 per cent for polarization values greater than 0.1.

Fluorescence spectra were recorded in a spectrofluorimeter equipped with two Bausch and Lomb monochromators. The slits of the monochromator were set to give a band width of 3 mμ. The enzyme aspartate aminotransferase from pig heart was purified according to the method of Sizer and Jenkins (6). When

analyzed in the ultracentrifuge Spinco at 56.000 RPM the enzyme showed a single component of sedimentation constant 5.3 S. The enzyme was labelled with 1-Dimethylaminonapthalene-5-Sulphonyl chloride (DNS) (7) and the average extent of labelling (1.1 - 1.5 DNS molecules/molecule of enzyme) was determined by measuring the fluorescence intensity of the conjugates at 520 mμ. The activity of the DNS-enzyme conjugate was found to be 95% that of the native enzyme when assayed according to the method of Sizer and Jenkins (6). Protein concentration was determined according to Lowry et al. (8).

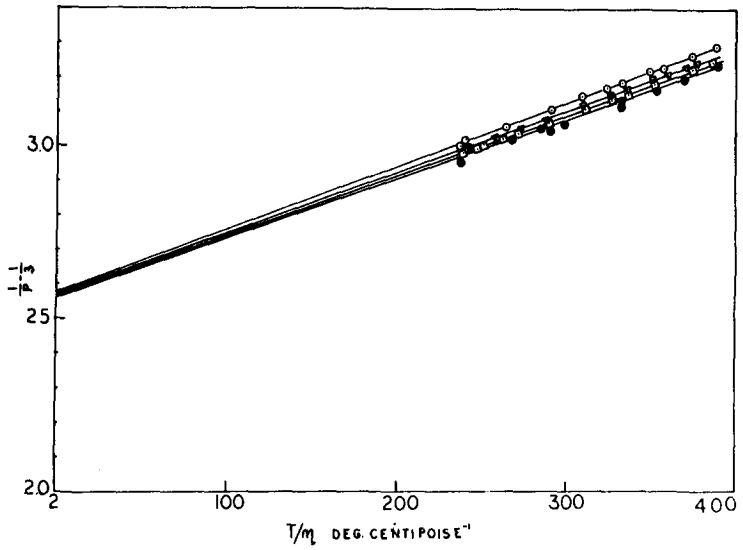

Figure 1. Polarization of fluorescence results of aspartate aminotransferase. Holoenzyme at pH: 7.8 ● and pH: 5.2 ⊡ Apoenzyme at pH: 7.8 ▽ and pH: 5.2 ⊙ . T/η was changed by increasing or decreasing the temperature of the solution.

The polarization of fluorescence properties of the conjugates were examined over the pH range 5.2 - 7.8. Figure 1 shows a plot of $\frac{1}{P} - \frac{1}{3}$ vs. T/η for the DNS-enzyme samples at a protein concentration of 0.04 mg per ml. The plot is linear within experimental error for the range 5° - 35° C. and fits Perrin's equation. Taking τ , the lifetime of the excited state to be $1.2 \cdot 10^{-8}$ seconds (13), the rotational relaxation time at 25° C. for the

macromolecule in solution was found to be 150 nanoseconds (Table 1).

Table 1

Samples	pH	ρh^1	ρh^2	ρs^2
Holoenzyme	5.2	150	175	2.6
Holoenzyme	6	155	170	2.5
Holoenzyme	7.8	150	170	2.6
Apoenzyme[3]	6	140	170	2.6
Apoenzyme	7.8	150	175	2.6

[1] T/η was changed by increasing or decreasing the temperature of the solution.

[2] T/η was changed by addition of sucrose.

[3] The apoenzyme was prepared according to the method of Scardi et al. (12).

Although the parameter ρ_n appears to reflect the rotation of the entire macromolecular unit in solution, it should be noted that a complete analysis of the polarization data must take into account the contribution of the rotational freedom of the fluorescent dye to the measured polarization of the system. If the fluorescent dye attached to the protein has some degree of internal rotation, then one would expect a decrease in the polarization P. In order to assess the relative contribution of the rotational freedom of the fluorescent dye to the measured polarization P, the technique proposed by Gottbieb and Wahl (9) was applied to the polarization measurements. Accordingly the polarization P was determined at constant temperature (25° C) and the viscosity of the medium was changed by addition of sucrose. It was found that the plot of $\frac{1}{P} - \frac{1}{3}$ vs. T/η shows a downward curvature at T/η values lower than 80. This type of curvature may occur whenever more than one rotational relaxation time is present and could be detected when ρ_n is several orders of magnitude larger than the relaxation time of a short segment of the protein (ρ_s) to which the dye is loosely bound (10,11,9). Following the theoretical treatment proposed by Gottbieb and Wahl (9), the polarization results in sucrose were analyzed by equation (1).

$$\frac{1}{P} - \frac{1}{3} = \frac{1}{Po} - \frac{1}{3}\left(\frac{1}{\frac{q}{1 + K_1\frac{T}{\eta}} + \frac{1 - q}{1 + K_2\frac{T}{\eta}}}\right) \quad (1)$$

where $K_1 = 5.45 \cdot 10^{-4}$; $K_2 = 4.2 \cdot 10^{-2}$; $q = 0.80$

The most salient feature of the results summarized in Table 1 is that the rotational relaxation times of the native and resolved species are similar. In addition the longest relaxation time (ρ_η), which reflects the rotational motion of the protein at concentrations approaching those used in the enzymatic assays, is compatible with the value expected for a rigid macromolecule of 100.000 Mw. The shortest relaxation time component (ρ_s) may be tentatively assigned to a polypeptide of the protein since the volume of the corresponding rotating unit in solution is approximately thirty fold smaller than the predicted value for a subunit of 55.000 Mw. The origin of ρ_s is conjectural and it remains to be established whether the segmental motion is an intrinsic property of the enzyme or whether sucrose <u>per se</u> is responsible for the observed effect.

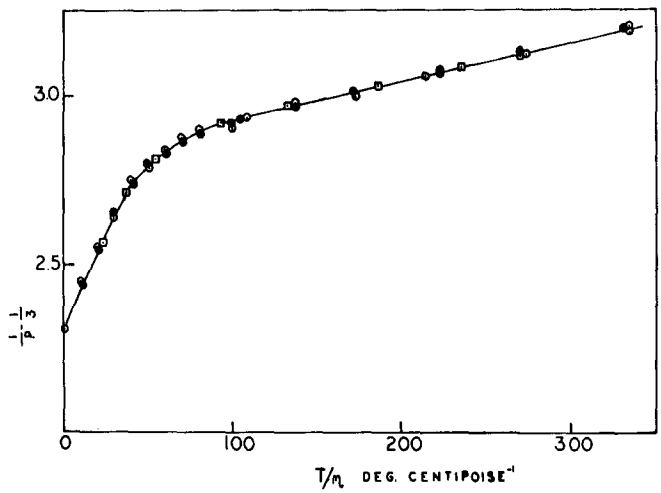

Figure 2. Polarization of fluorescence results of aspartate aminotransferase. Holoenzyme at pH: 7.8 ● and pH: 5.2 ⊡ . Apoenzyme at pH: 7.8 ⊙ Results obtained in sucrose solutions at 25° C.

ACKNOWLEDGMENTS

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REFERENCES

1. R. C. Hughes, W. T. Jenkins, and E. H. Fisher. Proc. Natl. Acad. Sci. U. S. 48, 1615 (1962).

2. C. Turano, A. Guartosio, F. Riva and P. Vecchini. J. U. B. Symposia on Pyridoxal Catalysis, Pergamon Press, London, 1963, p. 149.

3. W. T. Jenkins, D. A. Yphantis and J. W. Sizer. J. Biol. Chem. 234, 51 (1954).

4. J. E. Churchich and L. Harpring. Biochim. Biophys. Acta 105, 575 (1965).

5. G. Weber. J. Opt. Soc. Am. 46, 962 (1956).

6. J. W. Sizer and W. T. Jenkins in S. P. Colowick and N. O. Kaplan, Methods in Enzymology, Vol. 5, 1962, p. 677.

7. J. E. Churchich and F. Wold, Biochemistry 2, 781 (1963).

8. O. H. Lowry, N. J. Robrough, A. L. Farr and R. J. Randall. J. Biol. Chem. 193, 265 (1951).

9. Y. Gottbieb and P. Wahl. J. Chim. Phys. 60, 849 (1963).

10. G. Weber. Biochem. J. 51, 145 (1952).

11. R. Memming. Z. Fur Phys. Chem. 28, 168 (1961).

12. V. Scardi, P. Scotto, M. Daccurino and E. Scarano. J. U. B. Symposia on Pyridoxal Catalysis, Pergamon Press, London, 1963, p. 163.

13. R. R. Steiner and A. J. McLister. J. of Polym. Sci. 24, 105 (1957).