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Studies on the Changes in Protein Fluorescence and Enzymic Activity of Aspartate Aminotransferase on Binding of Pyridoxal 5′-Phosphate

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1. The α and β subforms of aspartate aminotransferase were purified from pig heart. 2. The α subform contained 2mol of pyridoxal 5′-phosphate. The apo-(α subform) could be fully reactived by combination with 2mol of cofactor. 3. The protein fluorescence of the apo-(α subform) decreased non-linearly with increase in enzyme activity and concentration of bound cofactor. 4. It is concluded that the enzyme activity/mol of bound cofactor is largely independent of the number of cofactors bound to the dimer. 5. The β subform had approximately half the specific enzyme activity of the α subform, and contained an average of one active pyridoxal 5′-phosphate molecule per molecule, which could be removed by glutamate, and another inactive cofactor which could only be removed with NaOH. 6. On recombination with pyridoxal 5′-phosphate the protein fluorescence of the apo-(β subform) decreased linearly, showing that each dimeric enzyme molecule contained one active and one inactive bound cofactor. 7. The results are not consistent with a flip-flop mechanism for this enzyme.

It has been suggested that the tryptophan fluorescence of oligomeric proteins will decrease non-linearly as successive ligand-binding sites on a given globular protein molecule are occupied by ligands that quench tryptophan fluorescence by long-distance radiationless transfer of electronic excitation energy (Holbrook, 1972). Non-linear quenching has been observed when fluorodinitrobenzene binds to avidin (Green, 1964), by di-iodotyrosines in serum albumin (Perlman *et al.*, 1968), Fe^{3+} in transferrin (Lehrer, 1969), and NADH in oligomeric dehydrogenases (McKay & Kaplan, 1964; Theorell & Takemoto, 1971; Holbrook *et al.*, 1972; Seydoux *et al.*, 1973). Linear quenching is expected and occurs when NADH binds to a monomeric dehydrogenase, octopine dehydrogenase (Luisi *et al.*, 1973), or when NADPH induces dissociation into monomers, as in mould glutamate dehydrogenase (Holbrook *et al.*, 1972). In those homo-oligomeric proteins so far examined, the relative quantum yields of molecules with i mol of bound ligand are found to be x^i (x is a geometric quenching factor; Holbrook *et al.*, 1972).

The yellow pyridoxal 5′-phosphate-containing enzyme, aspartate aminotransferase (EC 2.6.1.1), is dimeric and would be expected to show non-linear quenching during successive occupancy of the two cofactor sites on each molecule. However, the usual enzyme preparation (Martinez-Carrion *et al.*, 1965) contains electrophoretically and chromatographically separable subforms. Interpretable results would only be expected in experiments with homogeneous preparations of each subform. The nature of the differences between the subforms is still not clear. Banks *et al.* (1968) suggest that the subforms are due to stable conformations of otherwise identical subunits (Ovchinnikov *et al.*, 1973; Doonan *et al.*, 1974). Martinez-Carrion *et al.* (1970) suggest that the differences between the subforms reflect differences in the way in which coenzyme is bound. John & Jones (1974) suggest that the subforms reflect partial deamidation. These three explanations are not mutually exclusive.

In the present paper we report the isolation of the α and β subforms of aspartate aminotransferase and demonstrate that these holoenzymes have the spectral characteristics likely to result in quenched protein fluorescence due to energy transfer. We then remove the enzymically active cofactor from the proteins and study the linearity of changes in protein fluorescence as the apoprotein recombines with pyridoxal 5′-phosphate. From the linearity (or non-linearity) it is possible to deduce whether the dimeric enzyme with one cofactor per dimer has the same enzyme activity per bound cofactor as the fully ligated dimer.

Experimental

General

NADH, 2-oxoglutarate and pig heart malate dehydrogenase were purchased from C. F. Boehringer

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und Soehne G.m.b.H., Mannheim, Germany. Sephadex products were those of Pharmacia, Uppsala, Sweden. Hydrolysed starch (lot 257-1) was purchased from Connaught Medical Research Laboratories, Toronto, Canada.

Aspartate aminotransferase was assayed by the method of Karmen (1955) by using the linked system with malate dehydrogenase. One unit of enzyme oxidized 1 μmol of NADH/min at 25°C. Protein concentrations of the apo- and holo-enzymes were determined by measurements of E_{280} in 0.1 M-sodium phosphate buffer at pH7.4, by using the extinction coefficients of Banks *et al.* (1968). A molecular weight of 93000 (Feliss & Martinez-Carrion, 1970) was used in all calculations.

Pyridoxal 5'-phosphate solutions were standardized by using the extinction coefficient of 4900 litre $\cdot$ mol^{-1} $\cdot$ cm^{-1} at 385nm in 0.1M-sodium phosphate, pH7.0 (Storvick *et al.*, 1964). Absorption spectra were measured on a Perkin–Elmer 402 Ultraviolet–Visible Spectrophotometer, and absorption measurements were made on a Hilger–Gilford recording spectrophotometer equipped with a Smith's Servoscribe chart recorder or on a Zeiss PMQII spectrophotometer.

The pyridoxal 5'-phosphate content of the enzyme was determined by the method of Marino *et al.* (1972). A value for ε_{388} of 6600 litre $\cdot$ mol^{-1}cm^{-1} was used for the extinction coefficient of pyridoxal 5'-phosphate in 0.1M-NaOH (Peterson & Sober, 1954).

Preparation of the enzyme

Pig heart cytoplasmic aspartate aminotransferase was prepared by the method of Martinez-Carrion *et al.* (1965) and stored as an $(NH_4)_2SO_4$ suspension. Fractionation of the enzyme into its subforms was carried out by a modification of the method of Martinez-Carrion *et al.* (1967) as described in detail in the legend to Fig. 1.

Preparation of the apoenzyme

The prosthetic group of the α- and β-aspartate aminotransferase subforms was removed by the method of Dixon & Severin (1968) except that the Sephadex column was equilibrated with the buffer required in subsequent recombination experiments. Because of the instability of the apoenzyme it was prepared as required and not stored as a freeze-dried preparation. The residual enzyme activity of the α apoenzyme without added cofactor was 2% of that of the holoprotein.

Titration of the apoenzyme with pyridoxal 5'-phosphate

A series of solutions of apoprotein (about 1 mg/ml) in 0.1M-Tris–HCl, pH7.4, was incubated overnight at 0°C with samples of a pyridoxal 5'-phosphate solution (1–2mM). Equilibration took 3h. Protein fluorescence (excitation at 295nm, emission through a Kodak–Wratten no. 18A filter) was then measured by using the split-beam differential fluorimeter described by Holbrook (1972), except that the reference cuvette contained tryptophan and the reference photomultiplier was at 90° to the incident radiation. The instrumental configuration automatically corrected for inner filter effects caused by the added cofactor. These corrections were always less than 10%.

The amount of bound coenzyme was determined by making use of the spectral change at 362nm when the pyridoxal–enzyme is converted into the pyridoxamine–enzyme by addition of sodium glutamate to a final concentration of 50mM in 0.1M-Tris–HCl, pH7.4. All results are corrected for any slight dilutions.

Results

Fractionation of aspartate aminotransferase on CM-Sephadex (C-50) consistently produced an elution profile similar to Fig. 1. The conditions employed were a slight modification of the method of Martinez-Carrion *et al.* (1967), which produced four yellow protein peaks, three of which were not well resolved.

The first yellow peak to be eluted from the column (γ peak) appeared soon after application of the gradient and was closely followed by a larger peak (β). Although the β peak could be obtained free of contamination by the γ peak by taking a narrow cut, all samples of the γ peak were contaminated by the β peak. Enzymic assay of this double peak suggested that the γ peak did not contribute much to the enzymic activity. The last protein peak (α) to be eluted was well separated from the other two and could be pooled without danger of contamination.

The specific activities of the three pooled α, β, and γ fractions were 210, 120 and 50 units/mg respectively. The activity of the last (γ) fraction could have arisen partly or wholly from the observed contamination by the β fraction. Incubation of each fraction with pyridoxal 5'-phosphate had no effect on these specific activities.

The spectra of the three fractions at pH5 in distilled water are shown in Fig. 2. The α fraction had one absorption maximum at above 300nm (at 430nm), the β fraction had two absorption maxima (at 340nm and 430nm), and the γ fraction had a single absorption maximum at 340nm. The measured extinction coefficients for each subform at 340 and 430nm are given in the legend to Fig. 2.

A shift in the absorption spectra occurred as the pH was raised to pH7.4: the absorption maximum at 430nm of the α fraction shifted to 362nm, and a single absorption maximum at about 350nm appeared with the β fraction. After removal of the coenzyme from

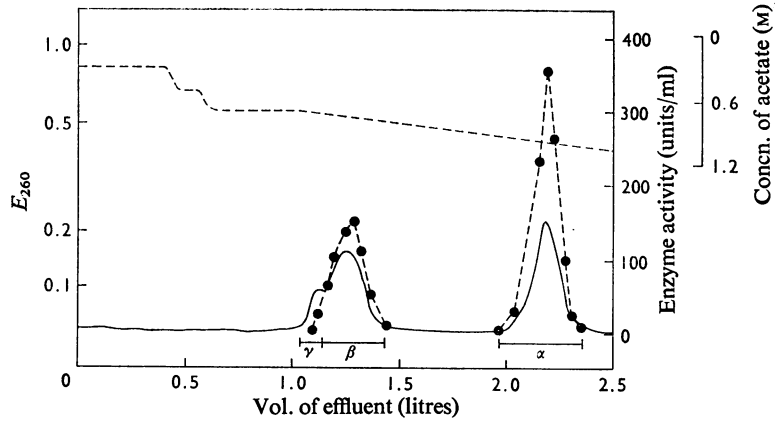

Fig. 1. *Fractionation of aspartate aminotransferase on CM-Sephadex*

Unfractionated enzyme (about 500mg) was centrifuged (8000*g*) down from $(NH_4)_2SO_4$ suspension, taken up in 20ml of water and either dialysed against 0.02M-sodium acetate, pH5.33, or passed through a column (4cm×32cm) of Sephadex G-50 (coarse) equilibrated in the same buffer. The enzyme was then applied to a column (4cm×32cm) of CM-Sephadex (C-50) equilibrated in the acetate buffer. The column was washed successively with 300ml of 0.04M-sodium acetate, pH5.33, 800ml of 0.06M-sodium acetate, pH5.33, and then a linear gradient of sodium acetate from 0.06M (pH5.33) to 0.11M (pH5.41), total volume 2 litres at a flow rate of about 35ml/h at 20°C. Each protein peak was pooled and the protein precipitated by addition of 430g of $(NH_4)_2SO_4$ to each litre and stored as a suspension at 4°C. ——, E_{260}; – – –, concentration of acetate (M); ●, enzymic activity.

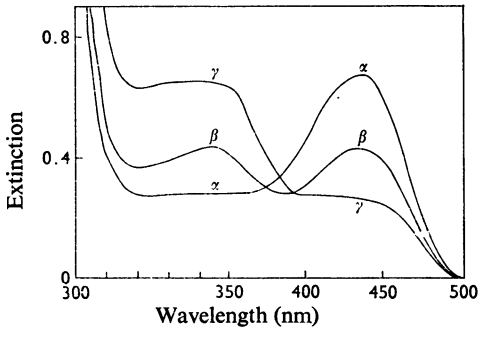

Fig. 2. *Spectra of the subforms of aspartate aminotransferase*

Spectra of α, β and γ subforms (5mg/ml) (separated by chromatography of the enzyme on CM-Sephadex) were measured after dialysis against water (pH5). The extinctions of 1% solutions of each subform were 0.56 (α), 1.0 (β) and 1.3 (γ) at 340nm, and 1.37 (α), 0.94 (β) and 0.57 (γ) at 430nm respectively. Note the change of scale on the abscissa.

the α and β subforms by the method of Dixon & Severin (1968), the α fraction was free of absorption above 320nm, whereas the β fraction had an absorption maximum at 340nm with a specific extinction coefficient of 0.79 for a 1% (w/v) protein solution.

The fresh α fraction gave only a single protein band on starch-gel electrophoresis with a discontinuous buffer system of Martinez-Carrion & Jenkins (1965) and Barrett *et al.* (1962). Traces of the γ subform were visible in the β subform. On prolonged storage the α subform produced β and γ subforms, and the β subform gave γ and more acidic subforms. The β subform did not revert to the α subform. These observations confirm those of John & Jones (1974) and are fully consistent with their proposal that deamidation accounts for the existence of the subforms of the enzyme.

Pyridoxal 5'-phosphate content of the α and β subforms

The spectrophotometric method of Marino *et al.* (1972) was used to remove and measure bound coenzyme as free pyridoxal 5'-phosphate. Some 1.8mol of pyridoxal 5'-phosphate was released/mol of the α subform in 0.1M-NaOH. Similar treatment of the β fraction before and after resolution resulted in values of 1.3 and 0.37mol of pyridoxal 5'-phosphate/mol of protein respectively. Thus only pyridoxal phosphate corresponding to one molecule per dimer is released by 0.1M-NaOH. The remaining extinction cannot correspond to 0.37mol of pyridoxal phosphate since it is not released by the alkali. Unfortunately, it is not possible unequivocally to calculate the number of mol of cofactor which remain without knowledge of an extinction coefficient for the 'inactive' cofactor.

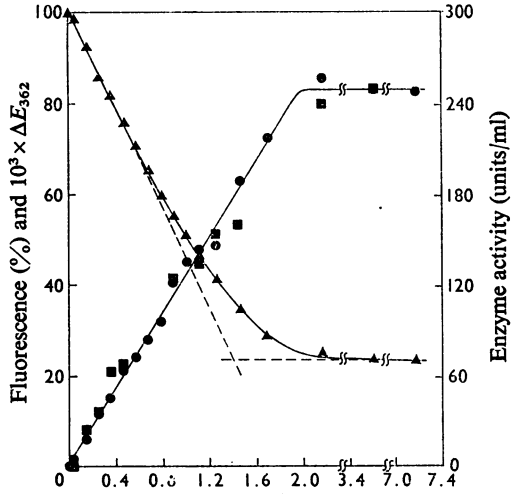

Fig. 3. *Titration of apo-(α-aspartate aminotransferase) with pyridoxal 5'-phosphate at pH7.4*

Portions of a stock solution of pyridoxal 5'-phosphate (1.25 or 2.5mM) were added to samples of resolved α-aspartate aminotransferase (3ml of 1.2mg/ml) in 0.1M-Tris–HCl, pH7.4, and the solutions were incubated for 12h at 4°C. ▲, Protein fluorescence after a 1:3 dilution with buffer; ■, absorbance change at 362nm when 20μl of 2.2M-sodium glutamate was added to the protein solution (1.2ml); ●, enzyme activity. All values were corrected for dilution. The broken lines represent tangents to the initial and final portions of each curve.

Titration of the resolved α and β fractions with pyridoxal 5'-phosphate

Only the α and β fractions were isolated in sufficient quantity for fluorescence titration experiments. Recombination of the resolved fractions with the coenzyme was measured by the change in protein fluorescence, the reappearance of enzymic activity and the spectral change which accompanies the conversion of the pyridoxal 5'-phosphate–enzyme complex into the pyridoxamine 5'-phosphate–enzyme complex.

With both resolved fractions the enzymic activity and the spectral change increased linearly with the amount of pyridoxal 5'-phosphate added, up to saturation values of 2mol of pyridoxal 5'-phosphate/mol of α fraction and 1mol of pyridoxal 5'-phosphate/mol of β fraction (Figs. 3 and 4). However, whereas the protein fluorescence of the resolved β fraction decreased linearly to a final value of 37% residual protein fluorescence, there was a non-linear decrease to 23.5% residual protein fluorescence with the α fraction. The regain of activity

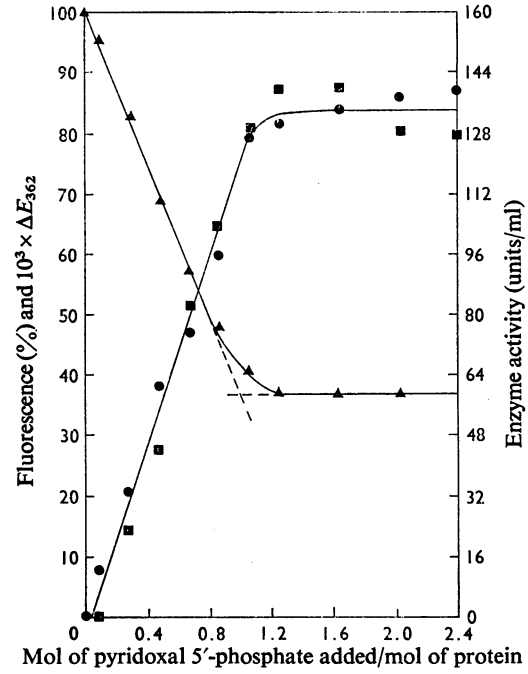

Fig. 4. *Titration of apo-(β-aspartate aminotransferase) with pyridoxal 5'-phosphate*

Portions of a stock solution of pyridoxal 5'-phosphate (0.825mM) were added to samples of resolved β-aspartate aminotransferase (3ml of 1.1mg/ml) in 0.1M-Tris–HCl, pH7.4, and the solutions were incubated at 4°C for 12h. ▲, Protein fluorescence after a 1:5 dilution with buffer; ■, absorbance change at 362nm when 25μl of 2M-sodium glutamate was added to the protein solution (1.0ml); ●, enzyme activity. All values are corrected for dilution. The broken lines represent tangents to the initial and final portions of each curve.

and decrease in protein fluorescence were slow processes and the above results are equilibrium values.

Titration of the resolved α fraction with pyridoxal 5'-phosphate showed that when all the coenzyme-binding sites had been filled the reconstituted enzyme had 38% of the protein fluorescence of the resolved enzyme at pH4.7, whereas at pH7.4 this value was 23%.

Discussion

Pyridoxal-containing proteins provide examples for the study of transfer of energy from a protein to its chromophoric cofactor. As protein-bound pyridoxal 5'-phosphate absorbs in the region 300–450nm, the actual absorption spectrum being characteristic of the particular protein and often pH-dependent, one of the conditions for long-range radiationless transfer

of energy, namely an overlap between donor emission spectrum (i.e. tryptophan fluorescence) and acceptor absorption spectrum (Förster, 1948), is satisfied by pyridoxal-containing proteins.

The dimeric aspartate aminotransferase is readily available from pig heart and was chosen with a view to testing the predictions of the theory of geometric quenching of protein fluorescence (Holbrook, 1972), since pyridoxal 5′-phosphate binds to the enzyme. Before any recombination studies could be carried out it was necessary to separate the electrophoretically distinct subforms of the enzyme, which have different spectral properties (Martinez-Carrion *et al.*, 1965, 1967). Although the method used for the separation on CM-Sephadex was only a slight modification of that of Martinez-Carrion *et al.* (1967), who obtained four poorly resolved protein fractions, the elution profile obtained by us was characteristic of three fractions. The first two fractions to be eluted from the column (γ and β fractions) were not always well resolved, but the last fraction (α) was consistently well separated from the earlier fractions.

Martinez-Carrion *et al.* (1967) correlated the enzymic activity of the various subforms with their absorption spectra. A high specific activity was associated with a strong absorption at 430nm at pH5.4. The specific activity decreased with a concomitant increase in absorption at 340nm. The absorption at 430nm (which shifted to 362nm at higher pH) was attributed to an 'active' form of the coenzyme, which could be reversibly removed and which participated in the transamination of amino acid substrates. The absorption at 340nm was thought to arise from an 'inactive' form of pyridoxal 5′-phosphate, which did not contribute to enzymic activity and could only be removed by concentrated base. However, no attempt was made to quantify the relative amounts of inactive and active coenzyme in the various forms of the enzyme.

The α subform isolated in the present study had a high specific activity of approximately twice that of the β subform, whereas the γ subform had the lowest specific activity. Because the β and γ subforms were not always well resolved, there was a strong possibility that the activity of the γ form was partly, if not wholly, due to contamination by the β form, and this deserves further study.

Specific-activity measurements on the fractionated enzyme suggested that perhaps the β subform comprised either half-active molecules, assuming that each site was enzymically independent, or a mixture of 50% completely active and 50% completely inactive molecules. The spectra of the two major subforms was consistent with this first theory (Fig. 2). At low pH the α subform had a strong absorption band centred around 430nm, characteristic of 'actively' bound pyridoxal 5′-phosphate, the γ form had an absorption maximum at 340nm and the β form had absorption maxima at 340 and 430nm. Assuming that the α and γ forms have only 'active' and 'inactive' pyridoxal 5′-phosphate, it is possible to calculate the theoretical specific extinction coefficients at 340 and 430nm for the β form if 50% of its coenzyme was 'active' and 50% 'inactive'. These theoretical specific extinction coefficients ($E^{1\%}_{340} = 0.93$, $E^{1\%}_{430} = 0.97$) are in reasonable agreement with the experimentally determined values ($E^{1\%}_{340} = 1.0$, $E^{1\%}_{430} = 0.94$).

Resolution of the enzyme removes only 'active' pyridoxal 5-phosphate (i.e. that enzyme-bound cofactor that is converted into pyridoxamine 5′-phosphate with excess of glutamate; Martinez-Carrion *et al.*, 1967). All the coenzyme could be removed from the α form, but identical treatment of the β form failed to remove the species with an absorption maximum at 340nm. The approximate specific extinction coefficient of the apo-(β subform) ($E^{1\%}_{340} = 0.79$) was comparable with half the value for the unresolved γ subform ($E^{1\%}_{340} = 1.3$), which again suggested that half the coenzyme in the β subform was bound inactively. The stepwise titrations of the apo-(α subform) and apo-(β subforms) of aspartate aminotransferase with pyridoxal 5′-phosphate (monitoring the regain of enzyme activity and regain of the ability of glutamate to convert the system into pyridoxamine phosphate) showed that 2 and 1 mol of coenzyme were reversibly bound at saturation to the respective proteins (Figs. 3 and 4). This agrees with the calculations based on extinction coefficients.

When a ligand binds to a homo-oligomeric protein and quenches protein fluorescence by long-distance radiationless transfer of electronic excitation energy [according to the Förster (1948) mechanism], then the protein fluorescence (F) will decrease with increasing fractional saturation of the ligand-binding sites (α). In a dimer with two sites, which are filled statistically, the decrease will be non-linear according to $F = [1-\alpha(1-x)]^2$, where x is a geometric quenching factor (Holbrook, 1972). When the sites are not filled statistically the form of the relationship between F and α will be linear: for complete positive co-operativity it will be a single straight-line decrease from the fluorescence of the apoprotein to the fluorescence of the di-ligated protein (since only these two forms are ever present in appreciable concentration); for complete negative co-operativity two straight lines will be observed corresponding to the change in fluorescence on sequentially saturating the first and second sites.

When apo-(α-aspartate transaminase) was titrated with pyridoxal 5′-phosphate there was a non-linear decrease in the protein fluorescence with increasing amount of cofactor added, to a value which was 23.5% of the starting value. The non-linearity was also evident from the point of intersection of the initial tangent to the protein fluorescence titration curve

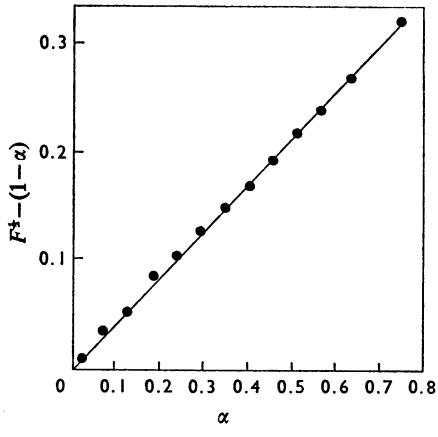

Fig. 5. *Demonstration that in the equation* $F^{\frac{1}{2}}-(1-\alpha) = x\alpha$, *x is a constant and is independent of* α

Results are taken from a titration of apo-(α-aspartate aminotransferase) with pyridoxal 5′-phosphate similar to Fig. 3.

and the asymptote corresponding to the protein fluorescence when the protein was fully saturated with coenzyme. Although both the enzymic activity and absorbance curves intercepted their respective lines corresponding to complete saturation at 2mol of pyridoxal 5′-phosphate/mol of protein, the intercept from protein fluorescence was 1.44mol of pyridoxal 5′-phosphate/mol of protein.

The theory of geometric quenching of protein fluorescence predicts that the ratio of the intercepts of the initial tangents to the protein fluorescence titration curve, and any physical quantity that responds linearly to the saturation of the protein with ligand, will be $(1-x^n)/n(1-x)$ for n independent and intrinsically identical binding sites with a geometrical quenching factor x (Holbrook *et al.*, 1972). In the case of α-aspartate aminotransferase, as n is 2 and the ratio of the intercepts is 0.72, x has a value of 0.44.

The theory also predicts that a plot of $F^{1/n}-(1-\alpha)$ against α will be linear with a gradient x, if x is constant and independent of α. Such a plot for α-aspartate aminotransferase is shown in Fig. 5; the plot is linear with a gradient of 0.42. Thus the quenching of the fluorescence of apo-(α-aspartate aminotransferase) can be interpreted in terms of radiationless transfer of energy from the protein to the coenzyme. The results with the transaminase are obtained by stepwise titration and are not sufficiently precise to be analysed by the curve-fitting techniques used previously for the dehydrogenases. However, the non-linearity allows the conclusion that uni-ligated dimer is present at appreciable concentrations at about the mid-point of the titration (Fig. 3). Since this Figure also shows that enzyme activity increases linearly with fractional occupancy of pyridoxal 5′-phosphate-binding sites, it must be concluded that the enzyme activity/bound pyridoxal 5′-phosphate is independent of whether one or both sites on the dimer are occupied by cofactor. This finding would be difficult to reconcile with suggestions that the two sites on the dimer interact by a flip-flop mechanism (Arrio-Dupont, 1972; Cournil & Arrio-Dupont, 1973).

This conclusion could be supported if it was possible to demonstrate that the β subform, with an average of one active cofactor/dimer, was a homogeneous population of dimers with only one active cofactor site (*a*), rather than a mixture of 50% fully active dimers and 50% fully inactive dimers (*b*). Possibility (*a*) would lead to a linear ($n = 1$) decrease in protein fluorescence with increasing saturation with cofactor (α). Possibility (*b*) would give a non-linear decrease for the 50% fully active dimers ($n = 2$) and no change for the inactive ones. The linear decrease observed in Fig. 4 supports possibility (*a*). Since the enzyme activity of the β subform (i.e. molecules with one active and one inactive form of the bound cofactor) is about 50% of the activity of the same weight of protein in the α subform (molecules with two active cofactors), this again argues that the activity at one pyridoxal 5′-phosphate-binding site is approximately independent of whether the second site on the molecule is occupied by active cofactor. Recent experiments with aldolase (Gibbons & Perham, 1974; Gibbons, 1974) showed that there were only small differences in the enzyme activity of an aldolase subunit depending on whether it was flanked by active or inactive subunits in the tetramer. Such small differences would not have been detected in the present experiments with aspartate aminotransferase.

Churchich (1965) has concluded that energy transfer between tryptophan and cofactor does not take place in transaminases, because no sensitized coenzyme fluorescence could be detected when pyridoxamine–aspartate aminotransferase was excited at 280nm. However, the native fluorescence of the bound coenzyme (excited at 340nm) has an exceptionally low quantum yield in comparison with other pyridoxamine–protein conjugates (Churchich, 1965), and any sensitized fluorescence would be so highly quenched as to be instrumentally undetectable. In any case it is not a necessary condition for radiationless transfer that the acceptor should fluoresce. A further indication that a major cause of the decrease in protein fluorescence on binding pyridoxal phosphate is the radiationless transfer of excitation energy by the Förster (1948) mechanism is that the degree of quenching depends on the overlap of the absorption spectrum of the acceptor with the fluorescence emission spectrum of the donor. At pH7.4 the protein fluorescence is lower than at pH4.7 where the absorption spectrum maximum at 430nm gives a less

favourable overlap integral. It would be impossible to exclude that a proportion of the decreased protein fluorescence on binding cofactor arose due to a direct interaction of the bound cofactor with a tryptophan residue at the active site. Such an interaction would lead to changes which were linearly proportional to the concentration of bound cofactor, as discussed previously for NADH-dependent dehydrogenases (Holbrook *et al.*, 1972). In this present case the close agreement of experiment with prediction (Fig. 5) suggests that the linear element in the quenching process is much smaller than that owing to long-distance energy transfer.

If the conclusions put forward above are correct they predict that the fluorescence of β-aspartate aminotransferase (experimentally determined as 0.37) should be (fluorescence of α subform)$^{\frac{1}{2}}$, i.e. $0.23^{\frac{1}{2}} = 0.48$. The discrepancy between theory and measurement is well within experimental uncertainty. In this respect it should be noted that such predictions are very sensitive to the presence of inactive protein in the resolved α subform. For example the presence of even 10% of an impurity which did not bind pyridoxal phosphate would decrease the value of x from $0.23^{\frac{1}{2}} = 0.48$ to $0.13^{\frac{1}{2}} = 0.33$. Amounts of non-renatureable protein in the 5–10% range would not have been detected experimentally. The measured fluorescence of the β subform (0.37) is considerably less than the value of 0.615, which would be predicted if there was a linear relation between protein fluorescence and molecules of bound cofactor.

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