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ACTIVE CENTRE EQUIVALENT WEIGHT OF GLUTAMATE DEHYDROGENASE FROM DRY WEIGHT DETERMINATIONS AND SPECTROPHOTOMETRIC TITRATIONS OF ABORTIVE COMPLEXES

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SUMMARY

The extinction coefficient of bovine liver glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3) at 280 nm has been redetermined from dry weight estimations as 0.93 $cm^2 \cdot mg^{-1}$. Glutamate and glutarate form abortive ternary complexes with the enzyme and NAD(P)H, the absorption spectra of which differ considerably from those of the free coenzymes and their binary enzyme complexes, and permit estimation of the equivalent weight of the active centre by spectrophotometric titration. A value of 57 000 is obtained.

Determinations of the molecular weight of the oligomer of bovine liver glutamate dehydrogenase (L-glutamate:NAD(P)$^+$ oxidoreductase (deaminating), EC 1.4.1.3), and the number and molecular weight of the subunits and the active centres, have presented difficulties. A concentration-dependent association of the oligomer is the main problem in determining the molecular weight and values of 250 000–400 000 have been reported. Recent light scattering[1] and sedimentation[2] data gave 300 000–340 000. Direct determination of the molecular weight of the subunit by light scattering, sedimentation and viscosity measurements in guanidine hydrochloride gave[1,2] 51 000–57 000, and gel filtration[3] in sodium dodecyl sulphate 45 000–50 000.

Estimates of the equivalent weight for NADH binding from fluorescence enhancement[4,5] and polarisation[4] measurements are in the range 48 000–55 000. Reports of wide variations with the degree of polymerisation of the oligomer[4,6] have not been substantiated[5]. The precision of such estimates is limited by the relatively large number of sites per molecule, the possibility of binding interactions between sites, the small fluorescence enhancement, and the non-linear relation of fluorescence intensity and coenzyme concentration. Evidence has also been adduced that the coenzyme binding sites detected by fluorescence measurements are not identical with the active sites[6,7].

Whilst the absorption spectrum of NAD(P)H in the binary complex with the enzyme differs only slightly from that of the free coenzyme, we have found that in the abortive ternary complex of enzyme, NAD(P)H and glutamate the difference is much

TABLE I

ESTIMATION OF SPECIFIC ABSORBANCE AT 280 nm BY DRY WEIGHT DETERMINATIONS

The enzyme preparation, 30 mg in 1.5 ml, was dialysed against 2 × 1 l 0.05 M phosphate buffer, pH 7.0, and then against 1 l for 24 h. After centrifuging, duplicate samples of 0.502 ml dialysate and enzyme were evaporated to dryness at 80°, heated at 110° for 2 h, and cooled in a vacuum dessicator over P_2O_5. After weighing, the heating was twice repeated to constant weight (± 0.02 mg). For extinction measurements at 280 nm, triplicate dilutions of 0.100 ml enzyme solution to 3.100 ml with 0.11 M phosphate buffer, pH 7.0, were used. Separate determinations with two enzyme preparations, A and B, were made, and the means of the duplicate and triplicate measurements are given.

Enzyme preparation:	*A*	*B*
Dry weight of dialysate (mg)	3.90 ± 0.04	3.86 ± 0.01
Dry weight of enzyme solution (mg)	13.79 ± 0.01	13.33 ± 0.03
Dry weight of protein (mg)	9.89	9.47
Protein concentration (mg/ml)	19.70	18.86
Absorbance at 280 nm	18.13 ± 0.03	17.67 ± 0
Extinction coefficient ($cm^2 \cdot mg^{-1}$)	0.920	0.937
Mean		0.93 ± 0.01

more pronounced, and allows direct spectrophotometric titration of the active centres.

Most previous estimates of molecular and equivalent weights were based on the extinction coefficient at 280 nm of 0.97 $cm^2 \cdot mg^{-1}$ for the enzyme estimated by OLSON AND ANFINSEN[8], who also found $A_{230\ nm}/A_{280\ nm} = 1.6$. Enzyme preparations obtained from Boehringer (London) as a crystalline suspension in $(NH_4)_2SO_4$ give values of 1.94 for this ratio, and by recrystallisation, chromatography and ultracentrifugation appear to be homogeneous[9,10]. A redetermination of the extinction coefficient based on dry weight estimations therefore seemed desirable. The mean value for two enzyme samples was 0.93 $cm^2 \cdot mg^{-1}$ (Table I). From recent tentative sequence data[11], the enzyme subunit contains 18 tyrosine and 3 tryptophan residues in a total of 506 amino acids, the calculated molecular weight being 56 100. From these data and the extinction coefficients for tyrosine and tryptophan, the extinction coefficient may be calculated[12] as 0.73 $cm^2 \cdot mg^{-1}$. For comparison with this figure, the experimental value of 0.93 $cm^2 \cdot mg^{-1}$ was corrected for light scattering by extrapolation of a plot of log wavelength against log absorbance in the 350–500-nm region. The corrected value was 0.89. The ratio of observed to calculated extinction coefficient of 1.22 is larger than that obtained for most other proteins[12].

In studies of the binding of $NAD(P)^+$ to glutamate dehydrogenase by equilibrium dialysis, we observed in controls of the enzymic assay used to estimate small concentrations of coenzyme with excess glutamate and glutamate dehydrogenase that the extinction coefficient at 340 nm for enzyme-bound NAD(P)H appeared to be significantly smaller than for free NAD(P)H. An abortive ternary complex of enzyme, NADH and glutamate was first detected[13] by the effect of glutamate on the fluorescence emission spectrum of enzyme-bound NADH. The difference spectra shown in Figs. 1 and 2 show that in these ternary complexes the nicotinamide absorption of both NADH and NADPH is decreased to a much greater extent than in the binary enzyme–coenzyme complexes, and the absorption maximum is slightly shifted to 345 nm. The extinction coefficient at 340 nm is 19% smaller than that for free

NAD(P)H. These findings are important both for the enzymic assays referred to previously, and for the interpretation of spectrophotometric studies of the NAD(P)$^+$–glutamate reaction with stoichiometric amounts of enzyme by rapid reaction techniques. As is shown in Fig. 3, the substrate analogue glutarate, which is a strong competitive inhibitor with respect to glutamate[14], forms an analogous ternary complex with a similar absorption spectrum. As will be described elsewhere, in the presence of glutarate the binding of NAD$^+$ and NADP$^+$ to the enzyme is greatly streng-

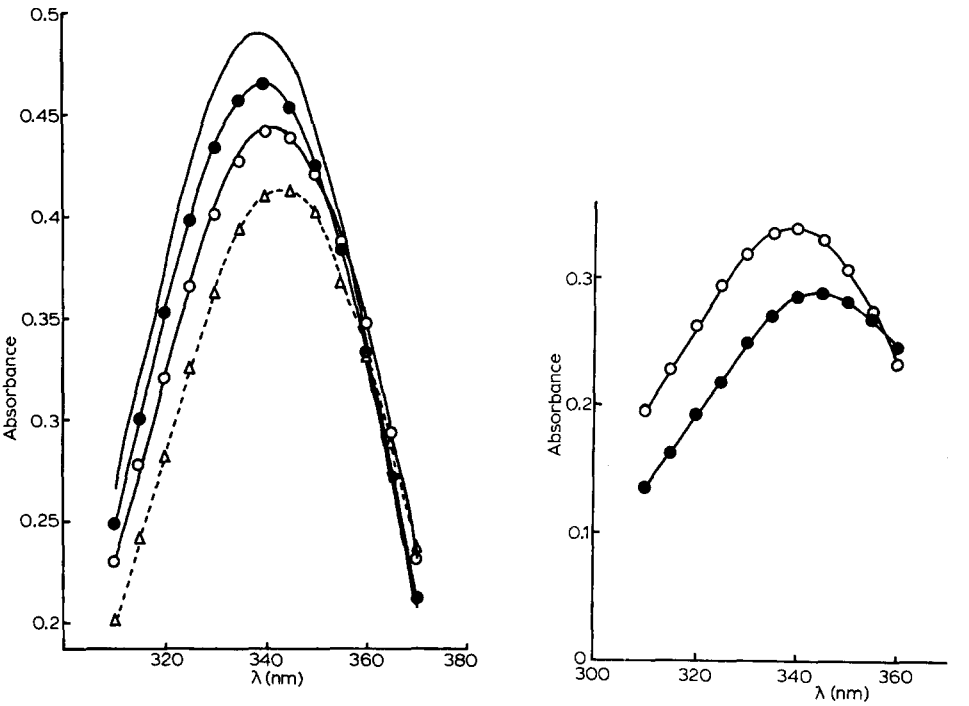

Fig. 1. Spectra of 79 μM NADH alone (———), in the presence of 4.24 mg/ml glutamate dehydrogenase (●—●), and in addition in the presence of 0.017 M glutamate (○—○ and 0.5 M glutamate (△----△). The buffer was 0.11 M phosphate, pH 7.0. The spectra are corrected for enzyme and glutamate absorbance.

Fig. 2. Spectra of 54 μM NADPH alone (○—○) and in the presence of 3.23 mg/ml glutamate dehydrogenase and 0.17 M glutamate (●—●) corrected for enzyme and glutamate absorbance. 0.11 M phosphate buffer, pH 7.0.

thened and exhibits negative interactions, consistent with the interpretation[9] of the complex kinetics of the NAD(P)$^+$–glutamate reaction.

The distinctive absorption spectrum of the ternary complexes of enzyme, NAD(P)H and glutamate permits spectrophotometric titration of the active centres. Even if the coenzymes can bind at sites other than the active centre, it is reasonable to suppose that the ternary complex characterises the active centres alone. Such titrations at 320 nm with both NADH and NADPH give sharp endpoints at 93 μM NAD(P)H with a protein concentration of 5.3 mg/ml calculated from the extinction coefficient of 0.93 $cm^2 \cdot mg^{-1}$. The value of 57 000 for the equivalent weight of the

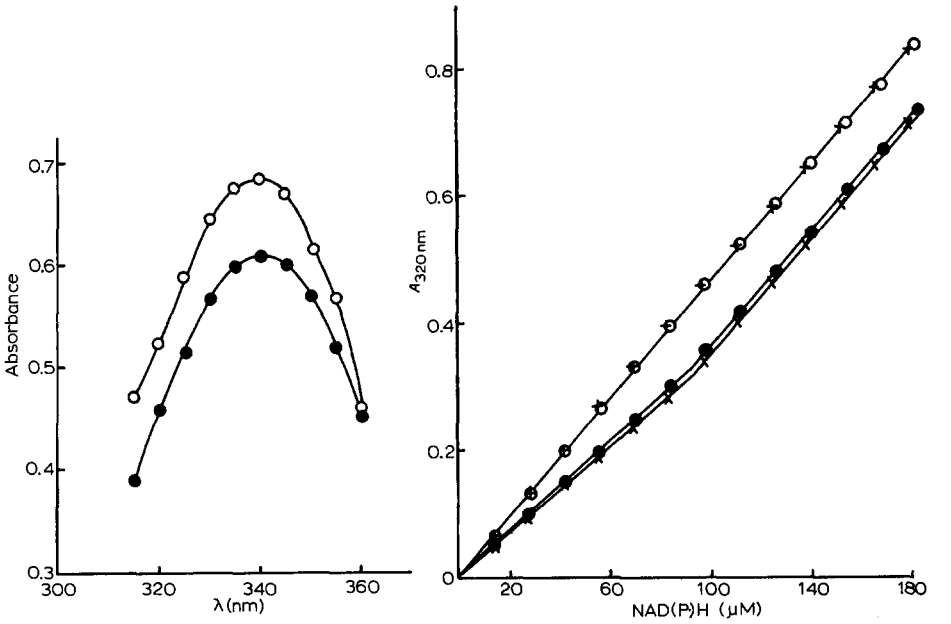

Fig. 3. Spectra of 110 μM NADH alone (○—○) and in the presence of 4.18 mg/ml glutamate dehydrogenase and 0.13 M glutarate, corrected for enzyme and glutarate absorbance.

Fig. 4. Spectrophotometric titration of glutamate dehydrogenase, 5.30 mg/ml, with NADH (●—●) and NADPH (×—×) in the presence of 0.17 M glutamate. The NAD(P)H was added in 5-μl aliquots to a total volume of 3 ml. Also shown are controls in which NADH (○—○) and NADPH (+—+) were added to 3 ml buffer containing 0.17 M glutamate, pH 7.0.

active centre is identical with the most recent estimate[2] of the molecular weight of the subunit from sedimentation equilibrium studies in guanidine hydrochloride, and establishes the existence of one active centre per subunit.

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