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Some Properties of Cross-Linked Polymers of Glutamic Dehydrogenase†

Robert Josephs, Henryk Eisenberg, and Emil Reisler*

ABSTRACT: Polymers of glutamic dehydrogenase are shown to be stabilized by cross-linking with glutaraldehyde. The nature of the cross-linked material is dependent upon the conditions chosen for the cross-linking reaction and appropriate conditions yield cross-linked polymers which are enzymatically active. Gel filtration of such polymers results in separation of the cross-linked material into fractions of narrow size distribution with molecular weights as high as 3×10^6. The molecular weights of such fractions are invariant with concentration in contrast with the behavior of the native enzyme. Acrylamide gel electrophoresis in sodium dodecyl sulfate shows that the major fraction of the cross-links bridge regions within a single molecule rather than between molecules. Kinetic parameters for the enzymatic reaction determined for fractions of different molecular weights show that enzymatic activity of the fixed enzyme is independent of the degree of polymerization.

Glutamic dehydrogenase is known to undergo polymerization (Sund, 1968) to form linear polymers (Eisenberg and Tomkins, 1968). The reacting species are in rapid equilibrium and their size distribution depends upon the concentration of enzyme present. At low protein concentration (less than 0.05 mg/ml) the monomer (mol wt 320,000) is by far the predominant species and, as the concentration is raised, progressively higher *n*-mers are generated; under appropriate conditions linear polymers up to 15–20 molecules long can be formed (for summaries, see Josephs *et al.*, 1972; Eisenberg, 1971). The distribution of sizes is not narrow but rather a broad spectrum of particle lengths is obtained (Reisler *et al.*, 1970). As an abundant and controversial literature will attest, these characteristics of the polymerization reaction introduce formidable interpretative difficulties in studies of the physical state of the enzyme.

An additional element of complexity is introduced upon examination of the relationship between the polymerization reaction and enzymic activity. This is exemplified by the observation that the presence of 10^{-3} M NADH and certain steroid agents or 10^{-3} M GTP cause nearly complete depolymerization and parallel inhibition of enzymatic activity. These and similar observations, when taken in conjunction, have led various workers to consider the relationship between enzymatic activity and polymerization and the role the depolymerizing agents mentioned above may play in regulating the form and activity of glutamic dehydrogenase *in vivo* (see, for example, reviews by Frieden, 1963a,b; Stadtman, 1966; and Tomkins *et al.*, 1963, 1965). A direct attack on this question is difficult since enzymatic activity is generally measured at concentrations so low that essentially all the enzyme is dissociated into monomer.

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In the work to be described below we believe we have succeeded in separating the enzymatic function from the polymerization properties of the enzyme. This has been achieved by covalently cross-linking polymers with glutaraldehyde. Such cross-linked polymers retain enzymatic activity; kinetic parameters such as K_m and V_{max} for various substrates being only slightly changed or identical with those of the native enzyme.

In contrast, the physical properties of the cross-linked enzyme are markedly changed. Cross-linked polymers do not change their degree of association in response to changes in enzyme concentration or the introduction of the allosteric effectors mentioned above. Consequently, cross-linked polymers may be separated into fractions of narrow molecular weight distribution and both physical and enzymatic properties of these fractions may be studied as a function of molecular weight.

Materials and Methods

a. Materials. Bovine liver glutamic dehydrogenase (L-glutamate: NAD(P) oxidoreductase (deaminating), EC 1.4.1.3) was obtained from C. F. Boehringer, Germany, and purine and pyrimidine nucleotides were obtained either from Boehringer or Sigma. Other reagents were of analytical grade and double-distilled water was used in all experiments.

b. Kinetic Measurements. Kinetic studies were conducted on control (unmodified) samples of bovine liver glutamate dehydrogenase and on three fractions of cross-linked enzyme of molecular weight 0.48×10^6, 0.94×10^6, and 3.6×10^6. Only completely cross-linked enzyme samples, *i.e.*, samples which possessed no ability to dissociate or to polymerize, were used in kinetic experiments.

Initial rate measurements were made at room temperature in 0.05 M potassium phosphate buffer (pH 7.6) in 1-cm quartz cells (Olson and Anfinsen, 1952). Enzyme concentrations for the deamination of glutamic acid were 0.0033 mg/ml and for the amination of α-ketoglutaric acid 0.0016 mg/ml unless otherwise noted. The rate of reaction was followed by monitoring changes in NADH concentration, at 340 mμ, with a Zeiss Model PMQ 11 spectrophotometer.

c. Electron Microscopy. Electron micrographs were obtained using Phillips 300 and JEM 100 B electron microscopes.

Negative staining was carried out as described before using the criteria for field selection previously mentioned (Josephs, 1971). The size distribution of different fractions of fixed enzyme was determined by counting the number of individual molecules per polymer chain and molecular weight moments computed by the usual formulas.

d. Molecular weights were determined by light scattering in a Fica (Paris, France) light scattering photometer as previously described (Eisenberg and Tomkins, 1968). The molecular weights of cross-linked enzyme in column effluents were estimated by monitoring the scattering at three angles only (135, 90, and 45°). The complete angular dependence of scattering (between 30 and 150°) was determined for fractions which were also examined by electron microscopy to derive molecular weights and weight distribution by direct particle counting.

Results

Fixation of Polymers of Glutamic Dehydrogenase with Glutaraldehyde. Addition of glutaraldehyde to protein solutions results in the formation of covalently cross-linked products (Korn *et al.*, 1972). At concentrations of the cross-linking agent approaching that commonly used for histological purposes, about 1%, the major product is an insoluble gel which contains over 90% of the protein originally present. This type of material is not readily amenable to physical characterization.

A soluble form of cross-linked polymer may be obtained under somewhat different reaction conditions. The rationale for choosing these is predicated upon the assumption that the gel is a product of interpolymer cross-links, the formation of which could be reduced by low protein concentrations and by minimizing the molar excess of glutaraldehyde over protein. For instance, gel formation occurred when cross-linking was carried out at an enzyme concentration of 6 mg/ml and a glutaraldehyde concentration of 1%, representing a molar excess of glutaraldehyde of over 5000-fold. If the concentration of both cross-linking agent and glutamic dehydrogenase is reduced then cross-linking takes place without formation of detectable insoluble products.

Preliminary experiments indicated that appropriate experimental conditions for cross-linking consisted of a protein concentration of the order of 1.0 mg/ml in the presence of a 50–100-fold molar excess[1] of glutaraldehyde. Unfortunately, because of the equilibrium nature of the polymerization reaction, only very short polymers, consisting of but a few monomeric units persist at such low protein concentrations (at 1.0 mg/ml the weight average degree of polymerization is less than 3). A much higher degree of polymerization may be achieved at low protein concentrations by addition of toluene or benzene (to saturation conditions) to the enzyme solution (in the presence of toluene a solution containing 1.0 mg/ml of protein has a weight average degree of polymerization of above 10). Such preparations have full enzymatic activity and are indistinguishable from native enzyme except by their greatly enhanced polymerization (Reisler and Eisenberg, 1970, 1972). Thus formation of long cross-linked polymers may be carried out in the presence of toluene at low protein and glutaraldehyde concentrations.

Cross-linking carried out under the conditions described above occurs slowly and up to a week is required at 4° for completion. The progress of the reaction is followed by measuring the "dissociability" of the polymer. For the system we are considering here this may be accomplished comparing the change in reduced light scattering[2] at 90° of partially fixed and unfixed solutions upon dilution with buffer not containing toluene. The ratio (multiplied by 100) of the specific scattering $(c_2I_1/c_1I_2) - 1$, for partially fixed and native enzyme, is referred to as dissociability; dilution factor c_1/c_2 was equal to 5. Unfixed solutions of glutamic dehydrogenase show a large reduction in the reduced scattering whereas completely fixed solutions show no change at all.

This formulation defines the dissociability of unfixed or native polymers as equal to 100 and for fixed enzyme 0; for partially fixed polymers the dissociability will have some intermediate value depending upon the extent of fixation.

The dissociability provides a convenient means for following the fixation reaction and determining optimal conditions for the reaction. Fixation proceeds more rapidly at higher molar excesses of glutaraldehyde and higher concentrations of protein. Molar excesses of glutaraldehyde between 25:1 to 125:1 were used; optimum results were obtained with a ratio of 75:1,

[1] These ratios are calculated on the basis of glutaraldehyde and enzyme monomer.

[2] The reduced scattering is the ratio of the observed scattering intensity I to the concentration c.

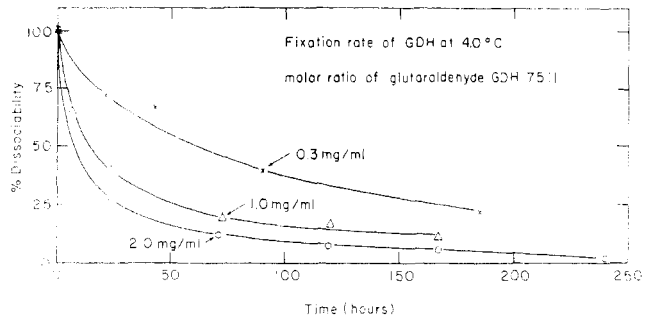

FIGURE 1: Plot of the dissociability (see text) of glutamic dehydrogenase as a function of time with molar excess of glutaraldehyde fixed at 75:1 and protein concentration varied as indicated.

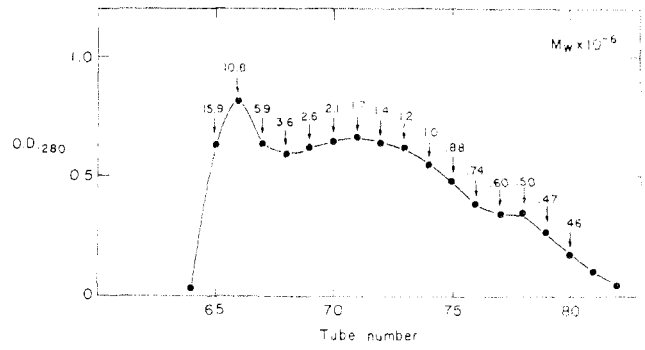

FIGURE 3: Chromatogram of fixed glutamic dehydrogenase fractionated on Sepharose 6-B. The molecular weights ($\times 10^{-6}$) of the fractions are given.

at 1.0 mg/ml of protein. At concentrations of protein much below 1 mg/ml (Figure 1) or much lower glutaraldehyde to protein molar excesses fixation is not complete even after 10 days.

The upper limit for the concentration of protein and glutaraldehyde is determined by the amount of interpolymer cross-linking considered acceptable. A rough estimate of this can be obtained by measuring the molecular weight of the polymers during cross-linking, as shown in Figure 2. Under all conditions of cross-linking we observed a small initial drop and a subsequent rise in molecular weight of the polymers, the extent of which depends upon the concentrations of protein and cross-linking agent. We interpret the rise in molecular weight as being indicative of the formation of interpolymer cross-linking. Figure 2 indicates that at concentrations of about 1.0 mg/ml of protein and a molar excess of glutaraldehyde of 75:1 cross-linking proceeds with only a small rise in polymer molecular weight. At lower concentrations even fewer intermolecular cross-links appear to be formed but then the reaction takes an excessively long time to go to completion. It is necessary to strike a compromise between the speed of reaction and formation of undesirable side products. The studies to be described below were carried out on polymers fixed at a protein concentration of 1 mg/ml and a molar excess of glutaraldehyde of 75:1.

Physical Properties of Cross-Linked Polymers of Glutamic Dehydrogenase

a. Polydispersity. When solutions of glutaraldehyde-fixed polymers of glutamate dehydrogenase are examined by ultracentrifugation or light scattering, profound changes in their physical-chemical properties are observed. The most striking of these is that the concentration dependent association–dissociation reaction observed in solutions of native enzyme is no longer evident. The molecular weight of solutions of fixed enzyme remains constant even after dilution to the lowest concentrations at which molecular weights can be measured. Neither removal of toluene nor addition of 10^{-3} M NADH + 10^{-3} GTP results in any change of molecular weight whereas either of these operations results in very drastic reductions in molecular weight of unfixed solutions of polymers.

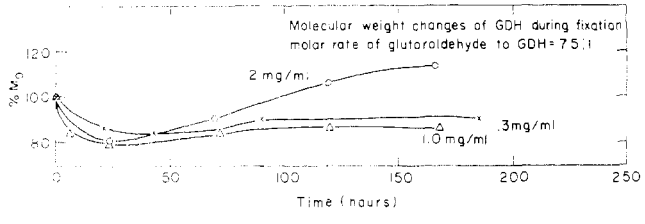

FIGURE 2: Changes of molecular weight of glutamic dehydrogenase during fixation at the indicated protein concentrations.

The length distribution of particles in solutions of native glutamate dehydrogenase is rather broad; at high degrees of association it is believed to approach the so-called "most probable distribution" which is characteristic of simple open-ended linear polymerization equilibria (Reisler *et al.*, 1970). For such a process the number average weight (M_n) is related to the weight average molecular weight (M_w) by $2M_n = M_w + M_1$ (Flory, 1953). It is not unreasonable to assume that after fixation polymer solutions will retain at least this degree of polydispersity, and perhaps even display a greater degree of heterogeneity due to formation of some interchain cross-links as well.

The high degree of polydispersity and the possibility that interchain cross-links form branched as well as linear species present formidable difficulties for the interpretation of physical-chemical data and for these studies it was necessary to fractionate the polymer solutions into fractions consisting of linear particles of narrow weight distribution.

b. Fractionation of Polymers. Efficient fractionation was achieved by applying a solution of fixed polymers (concentrated by ammonium sulfate precipitation) to a 5 × 100 cm column of Sepharose 6-B. A typical chromatogram along with the molecular weight of each fraction is shown in Figure 3. The chromatogram exhibits a peak in the void volume containing fractions of molecular weight between 5 and 15 million. This material was examined by electron microscopy and found to consist of polymers which had undergone multiple interchain cross-linking (Figure 4). The number of enzyme molecules aggregated in such particles may vary from 10 to 20 to much larger numbers of molecules.

Electron micrographs of fractions emerging just after the void volume in the molecular weight range of 3.6–1 × 10^6

TABLE I: Comparison of Molecular Weights ($\times 10^{-6}$) Obtained by Light Scattering and Electron Microscopy after One Pass over a 5 × 100 Sepharose 6-B Column.

Fraction	Light Scattering	Electron Microscopy
Cl-78	0.48	0.46
Cl-74	0.94	0.84
Cl-71	1.7	1.3
Cl-68	3.4	1.7

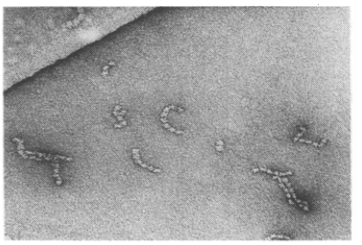

FIGURE 4: Electron micrograph of fixed glutamic dehydrogenase showing branched polymers consisting of 10–20 molecules.

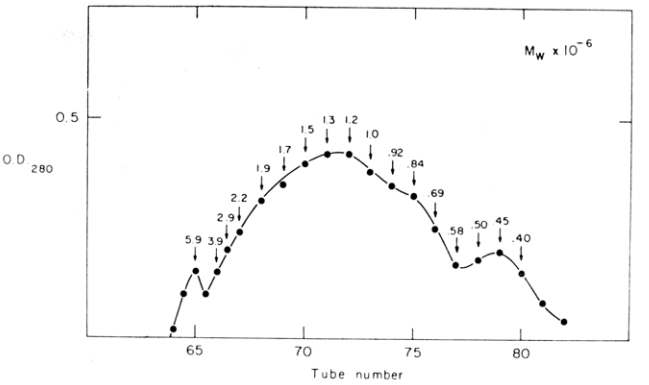

FIGURE 5: Rechromatography after removal of fractions eluting in the void volume.

display some, but progressively fewer, interchain aggregates as the position of fractions becomes further remote from the void volume. The presence of these aggregates is further indicated by comparing molecular weights measured by light scattering and by counting the number of enzyme molecules per polymer as seen in electron micrographs (Table I). The light-scattering procedure measures the weight average molecular weight including *all* polymer species whereas counting excludes the large nonlinear aggregates and therefore underestimates molecular weights to the extent that they are influenced by them. Electron micrographs of fractions eluting in the region of the chromatogram where molecular weights are less than 0.6×10^6 do not reveal the presence of aggregates and molecular weights determined by light scattering are in close accord with those obtained by electron microscopy.

A considerable reduction in the amount of nonlinear aggregates present in the high molecular fractions may be effected by discarding the fractions eluting in the void volume and subsequent rechromatography. After rechromatography the peak eluting in the void volume represents a much smaller proportion of the total mass than before (Figure 5). An alternative procedure which produces fractions containing even fewer aggregates consists of rechromatography of the fractions of molecular weight $3.6–1.0 \times 10^6$ and below 1.0×10^6 in separate batches.

Electron micrographs of such refractionated material reveal only occasional aggregates even when fields containing a relatively high density of particles are observed (Figure 6). Micrographs of these preparations taken at low magnification simultaneously display a large number of particles and are particularly useful for comparing the general appearance and relative frequency of aggregation in native and fixed enzyme. Higher magnification micrographs of polymers of different lengths confirm that the appearance of the fixed polymers is indistinguishable from that of untreated enzyme (see, for example, Josephs, 1971).

It is possible to separate the original heterogeneous mixture of fixed polymers into a spectrum of different molecular weight fractions consisting of linear polymers. We turn now to consider some of the properties of the fixed polymers.

c. Length Distribution of the Refractionated Polymers. Weight average molecular weights of different fractions of fixed polymers vary from as low as 350,000, corresponding to a large proportion of monomeric units, to as high as 2.0 million, the weight of a six unit particle. These molecular weights were obtained both from light scattering and from electron microscope histograms of the degree of polymerization (taking 320,000 for the monomer molecular weight). The concentration dependence of the molecular weight (from light scattering) of five fractions is shown in Figure 7; plots of the radically different concentration dependence of the molecular weight for native glutamic dehydrogenase in the presence and absence of toluene have been superimposed on this diagram for comparison.

The histograms of the number of monomer units per particle against particle frequency (Figure 8) show the size distribution characteristically obtained. The ratio of the number average molecular weight to the weight average molecular weight calculated from such histograms generally lies between 1.05 and 1.15 representing a very considerable improvement of the homogeneity of these fractions over the original solution. The weight average molecular weights determined by light scattering are in good accord with those obtained from histograms of the distribution particles per chain (Table II), providing additional verification of the success of the fractionation procedure.

d. Extent of Intersubunit Cross-Links. In considering the cross-linking process from a morphological point of view three types of cross-linking bonds may be distinguished. Those which bind one monomeric unit to its neighbor thereby maintaining the integrity of the polymers against dissociating agents such as GTP and NADH (intermolecular cross-links). Cross-links may also be formed between subunits within single molecules thus connecting subunits to each other (intersubunit cross-links). The third type of cross-link would bridge two residues within a single subunit (an intrasubunit link). It is difficult to assess, *a priori*, the relative abundance of these different types of covalent bonds since their main determinant is likely to be the spacial distribution of the reacting residues which is largely unknown. It is, however, possible to estimate the sum total intermolecular and intersubunit cross-linked material by acrylamide gel electrophoresis in sodium dodecyl sulfate (Carpenter and Harrington, 1972). Upon electrophoresis on sodium dodecyl sulfate gels, covalently linked subunits may be expected to migrate as distinct bands and one would therefore anticipate observing a spectrum of

TABLE II: Comparison of Molecular Weights (10^{-6}) Obtained by Light Scattering and Electron Microscopy after Rechromatography.

Fraction	Light Scattering	Electron Microscopy
E2-34	0.88	0.87
E2-28	1.44	1.26
E5-9	1.63	1.52
E5-8	1.96	1.71

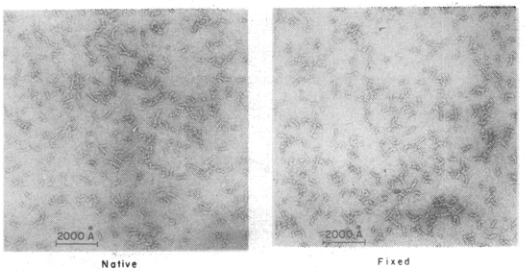

FIGURE 6: Electron micrographs comparing native and refractionated cross-linked enzyme.

bands corresponding to molecular weights which are integral multiples of the subunit weight of 53,500.

This is in fact borne out in Figure 9 which shows sodium dodecyl sulfate gel chromatograms for six fractions of fixed polymers of molecular weights from 4.2×10^5 to 3.2×10^6. The molecular weights of the bands are indicated by the vertical scale and the weight average molecular weight of each sample prior to dissociation with sodium dodecyl sulfate is indicated below the corresponding gel. The lowest molecular weight (4.2×10^5) corresponds to almost pure monomer while the highest weight is that of a ten-mer.

Several noteworthy features emerge from these gel patterns. (1) In all cases the major fraction of the material migrates in four bands corresponding to weights of 53,000, $2 \times 53{,}000$, $3 \times 53{,}000$, and $4 \times 53{,}000$, whereas uncross-linked samples yield only one band of 53,000; (2) there is a small amount of very high molecular weight material representing a minor fraction of the total which just enters the gel; (3) the characteristic band spectrum is invariant with the molecular weight of the fraction prior to application to the gel; (4) the most heavily staining band corresponds to the 53,000 subunit and the intensity of the other bands diminishes progressively as their molecular weight increases.

Initially one might have anticipated some correspondence

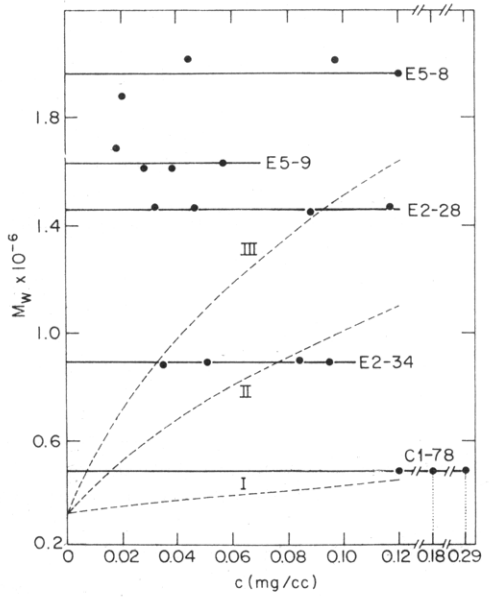

FIGURE 7: Concentration dependence of the molecular weight of five fractions of rechromatographed fixed enzyme. The molecular weights corresponding to each fraction are indicated. Similar plots for native glutamic dehydrogenase in 0.2 M phosphate buffer in the absence of toluene (curve I) and in solutions saturated with respect to toluene at 25° (curve II) and 10° (curve III) are shown for comparison.

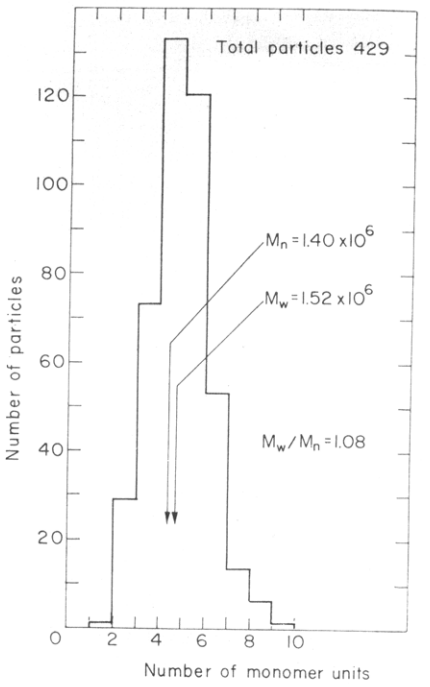

FIGURE 8: Typical histograms obtained for rechromatographed fixed enzyme. The histograms show the number of monomer units per particle of cross-linked enzyme.

of high molecular weight bands with the initial molecular weight of applied material. This is not observed and suggests that the major fraction of cross-links bridge regions within a single molecule. Particular support for this view derives from the band pattern of the lowest fraction, of molecular weight 4.2×10^5. The weight of this fraction corresponds primarily to that of the monomer and the band pattern must therefore arise from cross-links within the same molecule.

Enzymatic Properties of the Cross-Linked Polymers

Under standard assay conditions (Olson and Anfinsen, 1952), cross-linked glutamate dehydrogenase retains 80% of its enzymatic activity. This value, obtained for the completely fixed enzyme, is independent of the molecular weight of the examined sample. Because the enzymatic reaction is a complex one whose kinetic profile is sensitive to assay conditions, the above value is of small significance unless accompanied by study of kinetic properties of the modified enzyme under a variety of assay conditions. In order to define the enzymatic activity of the cross-linked enzyme, and particularly its behavior as a function of the number of enzyme molecules per

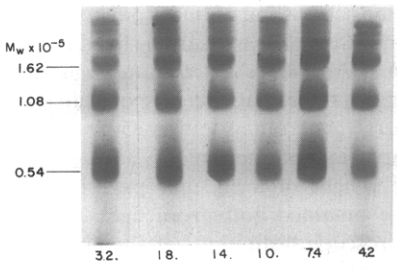

FIGURE 9: Sodium dodecyl sulfate acrylamide gels for six fractions of fixed enzyme. The molecular weights of the bands are indicated on the vertical scale and the molecular weight of the sample prior to dissociation with sodium dodecyl sulfate is indicated below each gel.

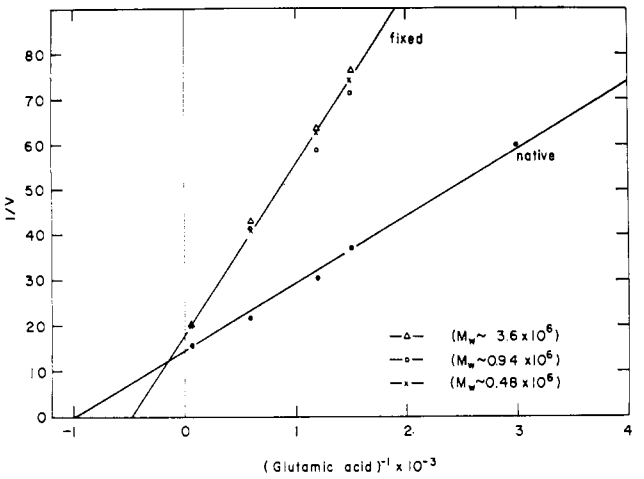

FIGURE 10: Double reciprocal plots of initial velocity with respect to glutamic acid concentration. The plots compare the activity of three fractions of fixed enzyme (molecular weights are indicated in the figure) with that of native enzyme (NAD 0.43 × 10^{-3} M).

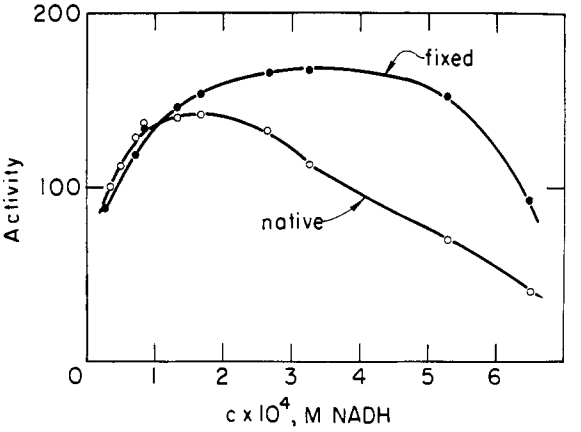

FIGURE 11: Plot comparing inhibition of activity of native and fixed enzyme by high concentrations of NADH: α-ketoglutarate 8.3 × 10^{-3} M; NH_4Cl 2 × 10^{-2} M.

chain, we have evaluated the kinetic parameters K_m and V_{max} for its several substrates and coenzymes for three fractions of cross-linked enzyme of molecular weight 0.48 × 10^6, 0.9 × 10^6, and 3.6 × 10^6.

a. Glutamic Acid and NAD. Figure 10 represents the results obtained for glutamic acid in the form of Lineweaver–Burk plots. It shows an increase in the value of K_m for glutamic acid from 1.1 × 10^{-3} M for the native enzyme to 1.8 × 10^{-3} M for the cross-linked protein. The plot indicates a small difference in V_{max} as well, but the major effect is the reduced ability of the fixed enzyme to bind glutamic acid. This means that higher concentrations of glutamic acid are required to achieve the maximum velocity of the reaction but the catalytic "activity" of glutamate dehydrogenase is only marginally changed on cross-linking.

The same reaction on the same samples as in Figure 10 was also examined as a function of the concentration of coenzyme NAD. The reaction was followed over its linear region, *i.e.*, at nonactivating levels of NAD. K_m for NAD (1.1 × 10^{-4} M) remained unchanged on cross-linking. The apparent decrease in V_{max} between the native and the fixed enzyme in this case derives from the different values of K_m for glutamic acid.

That the three fractions examined showed identical specific activity with respect to both substrate and coenzyme indicates that the degree of association has no evident effect on the catalytic activity. In fact, under no conditions have we been able to detect any difference in enzymatic properties of glutamate dehydrogenase polymers of different lengths.

The difference between the effect of cross-linking on Michaelis constants of L-glutamic acid and NAD is in line with the suggestion that they bind to different subsites of the enzyme as defined by Cross and Fisher (1970), and it appears that the locus of the NAD binding subsite is at a position which is not affected by the cross-linking reagent.

b. NADH as Coenzyme. When assayed with NADH, the activity of both native and cross-linked glutamic dehydrogenase depends on the concentration of coenzyme. Figure 11 shows results of these measurements. Identical values were obtained for the three fractions of cross-linked enzyme examined.

At lower NADH levels both the fixed and the native enzyme are activated to the same extent, and it seems reasonable therefore to speculate, that both forms have similar values for K_m. This will be true provided the long extrapolation, based on reaction velocities at low NADH concentrations only, is allowed in the double reciprocal plot ($1/v$ *vs.* 1/[NADH]). Such a procedure, though often accepted for unmodified glutamate dehydrogenase, does not yield accurate estimates of K_m.

It is known for some time that high levels of NADH inhibit glutamate dehydrogenase activity (Frieden, 1959). In cross-linked enzyme there is a considerable loss of this inhibitory property of NADH. The enzymatic reaction is activated over a wider range of NADH concentrations and the diminished inhibition is shifted to concentrations of NADH above 0.5 × 10^{-3} M. These results were again independent of degree of association of the fixed enzyme.

Similar effects on NADH inhibition of glutamate dehydrogenase reaction were observed in previous work on chemical modifications of the enzyme (Bitensky *et al.*, 1965; Colman and Frieden, 1966; Goldin and Frieden, 1971).

c. Cross-Linking and Some of the Regulatory Properties of Glutamate Dehydrogenase. The effect of cross-linking on the inhibition[3] of glutamate dehydrogenase by GTP, in the presence of NADH, is shown in Figure 12. Following the cross-linking K_{GTP} increases fivefold, from 2.3 × 10^{-6} to 11.6 × 10^{-6} M, again yielding identical results for the three fractions under study. A similar increase in K_{GTP}, four- to fivefold, was observed on modification of 0.5 lysine group/subunit of glutamate dehydrogenase with trinitrobenzenesulfonic acid (Goldin and Frieden, 1971). At this extent of modification 90% of activity was preserved. Acetylation of glutamate dehydrogenase (Colman and Frieden, 1966) and its reaction with organic mercurials (Bitensky *et al.*, 1965) also produced substantial decrease in the sensitivity of the enzyme to inhibition by GTP.

In contrast to the results for GTP, K_{ADP} decreases from 1.1 × 10^{-5} to 0.52 × 10^{-5} M on cross-linking of the enzyme. The same behavior is observed irrespective of the molecular weight of the fixed sample. Concomitant with the decrease in the ADP dissociation constant the extent of activation with excess ADP decreases too. This again is in qualitative agreement with previous works (Bitensky *et al.*, 1965; Colman and Frieden, 1966; Goldin and Frieden, 1971).

[3] The inhibited reaction is the "reverse" reaction described in the preceding section (b).

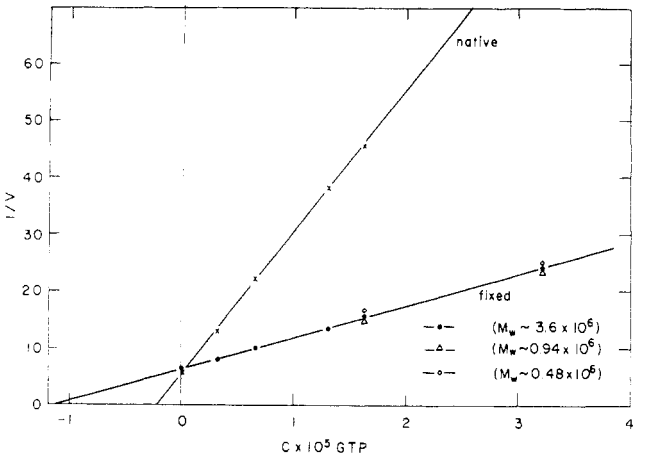

FIGURE 12: Plots of the reciprocal of the initial velocity as a function of GTP concentration. The plots compare the activity of three fractions of fixed enzyme (molecular weights are given in the figure) with that of the native enzyme (NADH 10^{-4} M, α-ketoglutarate 8.3×10^{-3} M, NH_4Cl 2×10^{-2} M).

Discussion

One of the most intriguing aspects of research on glutamate dehydrogenase are the attempts to correlate its functional and structural properties. For example, the relationship between the dissociating effect of GTP and NADH and their inhibition of glutamate dehydrogenase activity has stimulated speculations about the role of polymerization–depolymerization equilibrium in controlling the enzymatic functions of the enzyme. It has been suggested that some nucleotides and allosteric reagents can bind preferentially either to the polymeric or monomeric forms of glutamate dehydrogenase and in this way control and regulate the kinetic properties of the enzyme itself (Frieden and Colman, 1967).

Kinetic studies on binding of nucleotides to native enzyme were carried out at very low enzyme concentrations, *i.e.*, with fully dissociated protein. Thus, no direct investigation of the reciprocal relationships between (a) degree of protein aggregation and (b) nucleotide induced alterations of enzymatic properties and binding could have been conducted. By cross-linking of the enzyme we endeavored to separate the linear polymerization reaction from enzymatic functions with the goal of investigating each independently of the other.

Due to the cross-linking, enzymatic assays could have been done on linear polymers of glutamate dehydrogenase of molecular weight as high as 3.6×10^6. As shown for L-glutamic acid (Figure 10) the degree of polymerization of the cross-linked enzyme has no effect on its activity.

It is known for some time that the activity of glutamate dehydrogenase does not depend on its aggregation (Fisher *et al.*, 1962, 1965; Frieden, 1963a,b; Churchich and Wold, 1963; Bitensky *et al.*, 1965; Frieden and Colman, 1967; Reisler and Eisenberg, 1972), but now it has been possible to perform the activity tests on highly polymerized cross-linked samples of the enzyme under the standard assay conditions.

Of considerable interest is the effect of cross-linking on the kinetic effects of NADH. Acetylation of glutamate dehydrogenase or its reaction with trinitrobenzenesulfonic acid are believed to affect the interaction of NADH with a second, presumably nonactive, NADH site; interaction which normally results in the inhibition by excess NADH. In the modified enzyme this excess NADH inhibition is lost, though the binding ability of the coenzyme is not significantly different from that to the native enzyme. Similarly, GTP and ADP binding is not changed markedly upon the above modifications. It was suggested, therefore, that the coenzyme and the nucleotide sites are probably not altered by acetylation or trinitrobenzenesulfonic acid. This would imply, as proposed by Goldin and Frieden (1971), that the changes observed on modifications in K_{GTP} and K_{ADP} are related to the loss of NADH inhibition, rather than to desensitization of the enzyme toward these nucleotides. This conclusion was supported by the fact that at low extents of trinitrophenylation (0.5 lysine group/subunit) K_{GTP} was unhanged (compared to the native enzyme) when NADPH was used as a coenzyme.

The same explanation may be extended to the cross-linked enzyme. The changes in K_{GTP}, K_{ADP}, and NADH kinetics reported in this work are compatible with those reported by Colman and Frieden (1966) and Goldin and Frieden (1971). One can envisage a mechanism in which a conformational change is occurring upon binding of NADH to its second nonactive site. Such a conformational change will govern GTP inhibition and ADP activation of the enzyme. An indication for existence of a conformational change associated with binding of NADH to a second site was provided recently by Huang and Frieden (1972). Cross-linking of the enzyme, and in some cases its chemical modification, decreases its structural flexibility and may lead to a loss of ability to undergo conformational changes. For example, in the case of phosphorylase *b*, its cross-linking with glutaraldehyde abolished the homotropic cooperativity of AMP with this protein[4] (Wang and Tu, 1969). In the present case a loss of ability to undergo a conformational change would lead to an increase in the value of K_{GTP} and decrease in the value of K_{ADP} and explain the loss of polymerization ability of cross-linked glutamic dehydrogenase (if such a change is deemed necessary for the polymerization). A simpler explanation would be to assume that certain groups involved in the polymerization are masked by the glutaraldehyde. On the other hand, the absence of dissociation of cross-linked glutamic dehydrogenase on dilution or addition of GTP and NADH is easily explained by the introduction of covalent bridges between subunits of the enzyme.

In each case reported in this work the examined kinetic properties were independent of chain length indicating that the sites associated with enzymatic activity are not in the region which is masked upon polymerization. This point could not have been examined in earlier work on chemical modifications of glutamate dehydrogenase because of its subsequent irreversible dissociation or aggregation. By the same argument neither is the accessibility of the binding site for GTP or ADP (in the cross-linked enzyme) affected by the degree of polymerization; inhibition and activation, respectively, are independent of chain length.

This is to be compared with the work of Frieden and Colman (1967) who find that GTP, in the presence of NADH, binds preferentially to the monomeric form of the enzyme (mol wt 3.2×10^5), whereas ADP, in the presence of NADH, binds preferentially to the polymeric form. However, their argument in favor of tighter ADP binding, in the presence of NADH, to the aggregated enzyme is not well established. Kinetic studies (Colman and Frieden, 1966) indicated that the ADP dissociation constant is smaller for the acetylated enzyme. The authors though derive their conclusions from more accurate, direct binding studies (Frieden and Colman, 1967); these yield larger ADP dissociation constants for the

[4] According to Monod *et al.* (1965) conformational changes are prerequisite for cooperativity effects.

acetylated enzyme. But as only two samples have been examined (fully dissociated, acetylated and native, polymerized enzyme) it is impossible to decide whether the changes in ADP dissociation constant are due to acetylation or depolymerization. It is worth noting in this context, that ADP binding in the absence of NADH has recently been found to be independent of the state of association of glutamate dehydrogenase (Fisher *et al.*, 1972).

As for GTP, it is easy to accept its preferential binding, in the presence of NADH, to the monomeric form of glutamate dehydrogenase, both conceptually and on the basis of data presented. Our studies, though, do not reveal any changes in kinetic dissociation constant of GTP as a function of degree of polymerization of the cross-linked enzyme. It is conceivable that cross-linking causes the loss of heterotropic interaction (of GTP in the presence of NADH) either through partial masking of GTP subsite or by modification of subunit interactions. This point requires further clarification by direct binding measurements of GTP to native and cross-linked enzyme and perhaps to enzyme cross-linked in the presence of GTP and NADH. It seems also, that at this stage, in view of our knowledge of amino acid sequence and results of kinetic and modification studies on glutamate dehydrogenase, it should be possible to design experiments which could locate the sites of attack of different catalytic and regulatory reagents.

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