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Heat Denaturation of Bovine Liver Glutamate Dehydrogenase (38948)

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Denaturation of an enzyme by heat is accompanied by the loss of catalytic activity. At the two extreme positions, does the loss of 50% of the original activity reflect a 50% change in conformation of all the protein molecules so that the enzyme is less efficient in its functioning or does this loss of enzyme activity reflect a 100% change in conformation of 50% of the total population of protein molecules present so that these molecules are completely inactive catalytically? In the first case, all enzyme molecules are partially denatured and partially inactive catalytically (and perhaps altered in the capability to bind substrate or inhibitor competitive with substrate); in the second case, completely denatured and totally inactive enzyme molecules exist with wholly native and fully catalytically active protein molecules. The second case is called an *all or none* mechanism (1) or two step transition process (2–4). In the study reported here on heat denaturation of bovine liver glutamate dehydrogenase (L-glutamate NAD(P)$^+$ oxidoreductase (deaminating) EC 1.4.1.3), fractional loss of catalytic activity coincided with alteration in protein conformation for a corresponding percentage of protein molecules.

Material and Methods. Bovine liver glutamate dehydrogenase, NAD$^+$, and L-glutamic acid were obtained from Sigma Chemical Company. Isophthalic acid was purchased from Eastman Company. The crystalline enzyme was collected by centrifugation at 4° and subsequently dissolved in 0.1 *M* Na_2HPO_4 buffer previously adjusted to pH 7.8 with 5 *M* H_2SO_4. Stock solutions of enzyme (about 5 mg/ml) were kept refrigerated at 4°. Protein concentrations were estimated by biuret and Lowry methods (5). Water of 10^6 ohms resistance was used throughout the experiments. Enzyme assays were performed at 30° with use of a Beckman DU monochromator modified with Gilford 222, 2443A, 6039, and 3017 accessories, and connected to a Honeywell Electronik 19 strip chart recorder. Fisher serological water baths were used for maintaining temperature ± 0.5° during denaturation experiments. Protein fluorescence was measured with use of an Aminco-Bowman spectrofluorophotometer. Centrifugation of denatured protein samples was accomplished at 7° with a Sorvall SuperSpeed centrifuge and SS-1 rotor.

Results. Table I summarizes the effect of heat at 47° on the physical and catalytic properties of bovine liver glutamate dehydrogenase. Loss of catalytic activity at 47° measured at 47° was found to parallel the loss of activity of aliquots removed at 47° and subsequently assayed for enzyme activity at 30° (data not shown). Loss of enzyme activity paralleled loss of soluble protein (Table I) and diminution of protein fluorescence (activation, 300 nm; fluorescence, 350 nm; Fig. 1A). Complete loss of activity was observed at 3.5 hr. Enzyme activity once lost could not be regained by dilution and prolonged incubation at 30 or 0° even in the presence of L-glutamic acid or NAD$^+$. Denaturation corresponded to loss of enzyme activity, diminution of protein tryptophanyl fluorescence and concomitant increase in insoluble protein. The latter was removed from suspension by centrifugation. The loss of catalytic activity and the formation of insoluble protein was a first order reaction with respect to protein concentration. The Arrhenius energy of activation for denaturation at 45, 47, 50, and 55° was calculated to be 83,000 cal/mole (Fig. 1B).

The data of Table I showed that the loss of enzyme activity during the heating process did not occur from successive partial denaturation of all the enzyme molecules which were present and soluble at the beginning of the experiment. Indeed, the

TABLE I. ENZYME ACTIVITY AND PROTEIN CONTENT OF CENTRIFUGATE FRACTIONS OF BOVINE LIVER GLUTAMATE DEHYDROGENASE HEATED AT 47°.

Time of heating (min)	Enzyme activity[a] (μmoles NADH formed/min)	Protein content in enzyme assay (μg)	Specific enzyme activity (μmoles NADH formed/min/mg of protein)
0	0.051 (100)	11.3 (100)	4.51 (100)
10	0.039 (76.5)	8.5 (75.2)	4.59 (101.8)
20	0.033 (64.7)	7.3 (64.6)	4.52 (100.2)
40	0.025 (49.0)	5.4 (47.8)	4.63 (102.7)
60	0.017 (33.3)	3.8 (33.6)	4.47 (99.1)
80	0.011 (21.6)	2.5 (22.1)	4.40 (97.6)

[a] Five milligrams of bovine liver glutamate dehydrogenase in 50 ml of 0.1 *M* Na_2HPO_4-SO_4 buffer, pH 7.8, were stirred in a glass Sargent reaction vessel and heated at 47 ± 0.5° in a water bath. At different time intervals, 5 ml aliquots in 15 ml Corex centrifuge tubes were placed in ice for 10 min. The portions (0.1 ml) of the supernatant were assayed for protein content using the Lowry method (5) and for enzyme activity. The enzyme assay mixture contained 2.5 ml of 0.1 *M* Na_2HPO_4-SO_4 buffer, pH 7.8, 0.2 ml of H_2O, 0.1 ml of 60 m*M* L-glutamate, pH 7.8, and 0.1 ml of 18 m*M* NAD^+. The reaction at 30 ± 0.1° was begun by addition of 0.1 ml of supernatant. Velocity was determined as the change in absorbance per minute at 340 nm for the reduction of NAD^+. Values in parentheses represent the percentage of control value at zero time.

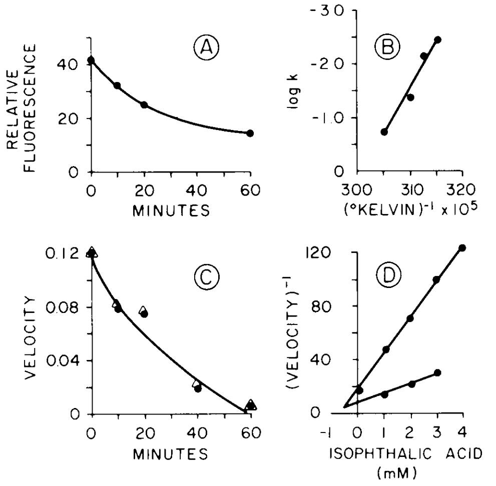

FIG. 1. Heat denaturation of bovine liver glutamate dehydrogenase. A represents relative fluorescence intensities of soluble protein fractions of Table I. B is an Arrhenius plot of first order denaturation rates as a function of the absolute temperature. Units of *k* are micrograms of inactive insoluble protein formed per minute. C depicts loss of enzyme activity of native (●) and collected "60" minute soluble enzyme (▲). Initial concentrations of both enzyme preparations were 100 μg/ml. Experimental conditions are given in Table I and text. D shows the effect of isophthalic acid on inhibition of "60" minute heat treated (Table I) glutamate dehydrogenase at two different concentrations of glutamic acid, 2 and 0.5 m*M*.

specific activity calculations showed that irrespective of the fractional loss of activity with concomitant change in the percentage of soluble protein, the remaining soluble and active enzyme molecules had full specific activity. A depression in the specific enzyme activity of the soluble and active enzyme molecules was not observed even after loss of about 80% of the original total enzyme activity. Therefore at 50% denaturation by heat of bovine liver glutamate dehydrogenase, one-half of the total population of enzyme molecules are fully active catalytically and probably unchanged in conformation and the other half of the protein molecule population is completely inactive catalytically with change in conformation (as evidenced here by change in protein fluorescence and by change in solubility).

In a second experiment to determine if the active enzyme molecules had been changed by the heating process, supernatants from 60 min (Table I) were concentrated using Schleicher and Schuell, Inc., collodion bags and filtration apparatus to give a protein concentration equivalent to the original solution (0.1 mg/ml). Comparison of loss of enzyme activity of control versus concentrated supernatant during heating at 47° for 90 min (Fig. 1C) showed no differences in the kinetics of denaturation. The two plots of inactivation as a function of time at 47° were superimposable. The heat treatment did not confer unusual resistance or susceptibility toward heat for the remaining "active" enzyme molecules. Dixon plots (Fig. 1D) of inhibition versus the concentration of isophthalic acid (an inhibitor competitive with L-glutamate for its binding site (6, 7) for heat treated enzyme (supernatant fraction of aliquot heated at 47° for 60 min, Table I) gave an inhibition constant of 0.5 m*M*, not different from that of unheated enzyme. This indicated that heat treatment had not altered the affinity of the substrate binding site for isophthalic acid in the remaining "active" enzyme molecules. These data together with those of Table I indicated that the "active" enzyme molecules which remained functional during fractional inhibition by heat denaturation were probably similar to the ones used originally to begin the experiment.

Discussion. Operationally, heat denaturation (45–55°) of bovine liver glutamate dehydrogenase appears to be an *all or none* (two step transition) process (1–4) whereby fractional loss of activity corresponds to a ratio between native, fully active enzyme molecules and denatured, completely inactive and insoluble enzyme molecules. These results are similar to the fractional loss of enzyme activity with concomitant denaturation of protein recorded for the inactivation of D-amino acid oxidase by sulfhydryl reagents (8).

Recently, Imoto, Fukuda, and Yagishita (9) showed by ion exchange chromatography that lysozyme digestion by pronase, nagarase, pepsin, and trypsin occurred by an *all or none* mechanism whereby lysozyme was digested in the denatured (unfolded) state. A similar two step transition has also been detected (10) through use of hydrogen–deuterium exchange in the denaturation of soy bean trypsin inhibitor. On the other hand, Tsung, Baldwin, and McPhie suggested (11) that transient forms may exist in ribonuclease denaturation by a "simple sequential model" as well as for other *all or none* processes of protein denaturation. Intermediate forms of partially inactivated enzyme were not observed here during heat treatment of glutamate dehydrogenase.

Summary. Heat denaturation of bovine liver glutamate dehydrogenase occurred at 47° with loss of enzyme activity and formation of inactive, insoluble protein. Fractional loss of catalytic activity coincided with alteration in protein fluorescence and solubility for a corresponding percentage of protein molecules. Operationally, at 50% denaturation, one-half of the total population of enzyme molecules is fully active catalytically and soluble and the other half of the protein molecule population is completely inactive catalytically and insoluble.

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