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ON THE ROLE OF TRYPTOPHAN RESIDUES IN THE MECHANISM OF ACTION OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AS TESTED BY SPECIFIC MODIFICATION

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Summary

A method is described to selectively modify one of the three tryptophan residues of the subunit of glyceraldehyde-3-phosphate dehydrogenase from yeast. As modifying agent dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide was used.

The residue which is modified by the procedure described has been identified as Trp-193. There are either one or two molecules of the modifying agent being added to this tryptophan side chain.

The modification apparently does not cause a detectable conformational change of the protein as judged from the methods employed.

However, the enzymatic activities in the dehydrogenase as well as in the esterase reactions are lost after the modification. It could be established that the modification rendered the enzyme unable to bind the oxidized coenzyme. Also the charge-transfer interaction between enzyme and coenzyme could no longer be observed.

Introduction

Not much is known about the role of tryptophan residues in the mechanism of action of enzymes. The tryptophan residues of lysozyme which have been shown to bind the substrate by hydrogen bond formation and by hydrophobic interaction with methyl groups [1] appears insofar to be an exception. Many authors have found that modification of tryptophan side chains alters and often abolishes the function of the polypeptide [2—14]. There is, however, no general rule as to how the alteration of the biological activity is mediated. Apart from participating in the catalytic process, tryptophan residues often

Abbreviations: SHNBB, dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide; HNB, 2-hydroxy-5-nitrobenzyl-; DTNB, disodium 5,5'-dithiobis-(2-nitrobenzoate).

appear to contribute to the conservation of the active conformation of proteins, probably by hydrophobic interactions [15—18]. This view is supported by measurements by various groups which have shown that tryptophan is one of the most hydrophobic amino acids [19—21]. Schellenberg proposed that tryptophan residues could play a part in the catalytic mechanism of dehydrogenases [22], but this view has not been confirmed.

In order to evaluate the role of tryptophan residues in the function of proteins one must have a method of selectively modifying the tryptophan residues under conditions which destroy neither the native conformation nor the enzymatic activity of the protein. These requirements are not satisfactorily met by most of the reactions of the tryptophan usually employed. The first aim of this paper is to describe the selective modification of the tryptophan-193 residue of glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) from yeast.

One characteristic feature of this enzyme is the formation of a broad absorbance band centered around 355 nm which occurs upon addition of the oxidized coenzyme, NAD^+, to the apoenzyme, the so-called Racker band [23]. Many authors agree that this band reflects the charge-transfer interaction between the coenzyme and a tryptophan residue of the protein [24,25]. (For opposing opinions see ref. 26).

We were able to show that modification of the tryptophan-193 abolishes the activity of the enzyme. This is due to the inability of the modified enzyme to bind NAD^+, as shown by various experiments. It can therefore be inferred that an intact tryptophan-193 is essential for the activity of this dehydrogenase.

Materials and Methods

Materials

Yeast was procured from commercial sources. SHNBB was supplied by Pierce Chemical Company; NAD^+, NADH, ATP, glycerate 3-phosphate, glyceraldehyde-3-phosphate diethylacetal, and phosphoglycerate kinase were obtained from Boehringer Mannheim GmbH, nicotinamide [*Carbonyl*-^{14}C]adenine dinucleotide from Buchler-Amersham, Braunschweig. All other reagents were analytical grade. For activity measurements, the biuret method, and the estimation of HNB groups an Eppendorf photometer (Netheler und Hinz, Hamburg) was used. Other photometric measurements were carried out with a Zeiss DMR 21, a Zeiss PM 4, and a Gilford 2400 S spectrophotometer. For reactions at constant pH a Radiometer TTT1 autotitrator was used. Liquid scintillation counting of ^{14}C activity was carried out with Bray's solution [27] in a Packard TriCarb Liquid Scintillation Counter. Amino acid analyses were performed with a BioCal BC 200 amino acid analyzer.

Preparation of the glyceraldehydephosphate dehydrogenase

The isolation and purification of the enzyme followed the procedure given by Kirschner and Voigt [28] except that all buffers used following the charcoal treatment contained 1 mM mercaptoethanol. The final recrystallization steps were carried out with protein concentrations of 10—20 mg/ml. The specific

activity of freshly prepared enzyme was 140—155 units/mg in the back reaction (glycerate-1,3-diphosphate + NADH). The $A_{280\,nm}/A_{260\,nm}$ ratio was greater than 2.10 indicating that virtually all coenzyme had been removed [28].

Assay conditions

The assay mixture contained 0.1 M triethanolamine · HCl, pH 7.6, 1.1 mM ATP, 1 mM EDTA, 0.23 mM NADH, 2 mM $MgCl_2$, and 10—100 μg phosphoglycerate kinase in a total volume of 3.0 ml [29]. The activity was calculated from the initial slope of the trace recorded at 366 nm. To obtain full enzymatic activity the enzyme solution was preincubated for 15 min at room temperature with 0.5 mM dithiothreitol and 0.1 mM NAD^+. The activity regained after this treatment decreased only by a few per cent per week [28,30].

Protein determination

The protein concentration of purified preparations of the apoenzyme was estimated using $A^{0.1\%}_{280\,nm} = 0.89$ [31]. The concentration of solutions of the modified protein could be measured using the biuret method since the absorbance of the hydroxynitrobenzyl chromophore does not extend into the region employed in the biuret assay. The factor given by Kirschner and Voigt [28] was used for the calculation of the protein concentration.

NAD^+ determination

NAD^+ concentrations were tested by enzymatic reduction with ethanol and yeast ethanol dehydrogenase. The assay mixture contained 0.2 M hydrazinium sulfate, 50 mM glycine buffer, pH 9.5, 3 mM EDTA, and 0.05 mg crystalline enzyme.

Enzyme modification

The crystal suspension of the apo-glyceraldehydephosphate dehydrogenase was centrifuged. The sediment was dissolved in a few ml of deionized water containing 0.1 mM EDTA. The solution was dialyzed against 50 mM triethanolamine · HCl, pH 7.5, containing 0.1 mM EDTA for at least 2 h at 0—4° C. Then the protein solution was made 0.2—0.25 mM (about 8 mg/ml) by addition of water, and a 7-fold molar excess of DTNB was added, with constant stirring for 15 min at room temperature. The pH was kept at 6.75 by addition of 0.1 M NaOH. From this point the protein solution was usually protected from light. To the SH-protected protein a 30-fold molar excess of SHNBB was added in three parts with stirring and the pH was held at 6.75. 20 min later an equal amount of SHNBB was added the same way. After 60 min the reaction mixture was centrifuged for 20 min at 20 000 × *g* to remove insoluble material which precipitated during the reaction. The remaining soluble contaminants were then separated from the modified protein by passing the supernatant through a column of BioGel P2, 200—400 mesh (5 × 11 cm). The column was eluted by 50 mM triethanolamine · HCl, pH 7.5. To the fractions containing the protein 20 mM mercaptoethanol (dissolved in a small amount of buffer) was added to remove the protecting thionitrobenzoate groups from the cysteine residues. The volume was then reduced by ultrafiltration through Amicon

PM 30 membranes, and the concentrated solution was purified by gel filtration through a Sephadex G-15 column (2.5 × 35 cm).

When the reaction of glyceraldehydephosphate dehydrogenase with SHNBB was carried out in the presence of NAD^+, a 60-fold excess of the modifying agent was added at once. The SH groups had not been protected, and therefore no estimation of the enzymatic activity after the modification was made, nor could the amount of HNB groups bound to the tryptophan residues be determined. The extent of the tryptophan modification was calculated from analyses of the intact tryptophan residues after the modification.

Estimation of the HNB groups attached to the protein

For HNB group determination, 0.5—1.0 mg of the HNB-glyceraldehydephosphate dehydrogenase was denatured in 9 M urea overnight at room temperature in the dark. The mixture was then applied to a Sephadex G-15 column (0.7 × 14 cm) which was equilibrated and eluted with 9 M urea solution. The protein fraction was dialyzed against deionized water. Upon addition of 0.05 ml 5 M NaOH the protein dissolved. It was transferred to the photometer cell; the HNB group concentration was calculated from the absorbance at 410 nm using $\epsilon = 1.85 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ [32]. An aliquot of the solution was then used to determine the protein concentration. In the preparative-scale experiment the gel filtration in urea solution was carried out using a 2 × 20 cm column.

Preparative-scale digestion

Digestion of the HNB-glyceraldehydephosphate dehydrogenase was accomplished by incubating the suspension of the urea-denatured and dialyzed protein with trypsin and chymotrypsin (3% each of the dry weight of the HNB-glyceraldehydephosphate dehydrogenase) at pH 8; 0.1 mM Ca^{2+} were added as well as a drop of $CHCl_3$ to prevent microbial growth. After 5, 20, 25, and 30 h the incubation mixture was recharged with the same amount of trypsin and chymotrypsin.

Separation of the tryptic/chymotryptic peptides

The digest was resolved on a Sephadex G-15 column (1.5 × 90 cm) which was eluted with 4% ammonia in water. The yellow fractions were further purified by paper chromatography on MN 2214 ff sheets (Macherey und Nagel, Düren). The solvent was butanol-2/formic acid/water (70 : 9 : 21, by vol.). Ninhydrin-positive zones which were turning yellow upon exposure to ammonia were eluted and subjected to electrophoresis on the same paper (60 min, 40 V/cm, 20 mA; buffer: pyridine acetate, pH 3.1). Ninhydrin-positive, yellow bands were eluted again and lyophilized. The fingerprint experiment was carried out on Whatman No. 3MM sheets. Electrophoresis buffer and chromatography solvent were as mentioned above.

Estimation of the SH groups

The protein was reduced with 1 mM dithiothreitol for 15 min at room temperature. The sulfhydryl reagent was then separated from the protein by a Sephadex G-15 column (1.5 × 20 cm) eluted with 50 mM triethanolamine · HCl buffer, pH 8.6, containing 0.1 mM EDTA. 0.7 ml of the protein fraction,

diluted to 18—20 μM (about 0.6 mg/ml) was rapidly mixed in a 1-cm cuvette with 0.01 ml freshly made DTNB solution in the same buffer (7 mM). The absorbance change at 405 nm was recorded. The blank contained the same components except the protein.

In the stopped-flow experiments, carried out with the Durrum rapid-scanning equipment, the final concentrations of the components in the mixture were the same.

Tryptophan estimation

Tryptophan concentrations were estimated according to Messineo and Musarra [33] except that the tryptophan concentrations ranged from 0 to 20 nmol and that the blank contained all components but tryptophan. The reliability of this method was tested by amino acid analysis following hydrolysis of the protein by 4-toluenesulfonic acid in the presence of tryptamine.

Active site titration

The apoenzyme was preincubated for 15 min at room temperature with 2 mM dithiothreitol in 50 mM triethanolamine · HCl, pH 7.8, in the presence of 0.1 mM EDTA. The excess SH reagent was separated from the protein by gel filtration immediately before the experiment. The enzyme concentration in the photometer cell was 9—27 μM (0.3—0.9 mg/ml). To 0.7 ml solution, 0.01 ml 50 mM *p*-nitrophenylacetate in acetonitrile was added. The mixing was completed within about 1 s. The absorbance at 400 nm was recorded. Under the same conditions a blank was run without enzyme.

Coenzyme binding studies using gel filtration

A column (1.5 × 30 cm) of Sephadex G-50 fine was equilibrated with 67 mM phosphate buffer pH 7.5 containing 62 μM NAD^+. 5—7 mg protein were applied to the column in each run. The eluate was monitored by recording the absorbance at 254 nm to locate both the positive and the negative peaks.

Equilibrium dialysis

Equilibrium dialysis was carried out in Visking dialysis bags. The volume of the protein solution was approx. 0.5 ml. Dialysis bags containing native or modified glyceraldehydephosphate dehydrogenase, respectively, were dialyzed in the same vessel against each of the desired concentrations of [^{14}C]NAD^+. After 40 h the radioactivity of the solutions was tested. It was shown that appropriate amounts of HNB groups quenched the counting rate by about 3%. NAD^+ concentrations were determined by the ethanol dehydrogenase reaction.

Results

Incorporation of HNB groups

The reaction of the glyceraldehydephosphate dehydrogenase with SHNBB resulted in the incorporation of 1.13—1.80 covalently bound HNB groups per subunit depending on various experimental parameters. Estimation of the tryptophan residues revealed that modification of the apo-glyceraldehydephos-

phate dehydrogenase under the conditions described results in the loss of one of the three tryptophan residues. Tryptophan analyses of the HNB-glyceraldehydephosphate dehydrogenase gave values of 1.9—2.1 tryptophan per subunit as compared to 2.9—3.0 for the native enzyme.

When the modification was carried out in the presence of 0.59 mM NAD^+ the tryptophan determination revealed the loss of only 0.5 residue per subunit.

Activity of the modified glyceraldehydephosphate dehydrogenase

Modification of the apo-glyceraldehydephosphate dehydrogenase with SHNBB largely abolishes the enzymatic activity. In most experiments a residual activity in the back reaction of 0—10% remained. The K_m of the modified preparations (0.25—0.29 mM NAD^+) were very close to the values of the native enzyme (0.18 mM NAD^+) [34]. This suggests that the enzymatically active protein is unmodified glyceraldehydephosphate dehydrogenase rather than a modified species.

SH groups

Since the alkylating agent SHNBB is known to react with sulfhydryl groups as well as with tryptophan side chains [32,35] the reactive cysteine residues had to be protected during the alkylation reaction, and it was necessary to show that the protection of the SH groups was fully reversible.

Incubation of the SH-protected enzyme with 20 mM mercaptoethanol did in fact restore full enzymatic activity. Comparing the time course of the reaction with DTNB of the native and the modified glyceraldehydephosphate dehydrogenase, respectively, proved that no SH group had reacted irreversibly during the modification and that the reactivity and the accessibility of both groups were similar in the two proteins. The conversion of the more reactive SH group was completed within 1 min whereas it took about 10 h for the more inert SH to react quantitatively. The stoichiometry of the reaction showed in either case one SH group per subunit to be involved (Fig. 1). When the fast reaction with DTNB was observed using the stopped-flow equipment, the reaction rate of the native glyceraldehydephosphate dehydrogenase was higher compared to the HNB-glyceraldehydephosphate dehydrogenase.

Disc electrophoresis

Because the modification of the protein carried out in this investigation conserves the net charge of the protein, the migration in disc electrophoresis should reflect the shape of the molecule. No difference was observed between the native and the modified proteins (Fig. 2).

Identification of the labeled residues

Proteolytic digestion by trypsin and chymotrypsin requires incubation times of up to 25 h. Separation and purification of the peptides bearing the HNB label, as described in the methods section, yielded the peptides listed in Table I. Further yellow fractions containing ninhydrin-positive material appeared in trace amounts too small to be purified. Their appearance may be due to incomplete hydrolysis of the cleavage sites. The HNB groups were evaluated by their absorbance at 410 nm at alkaline pH. The amino acid composition was

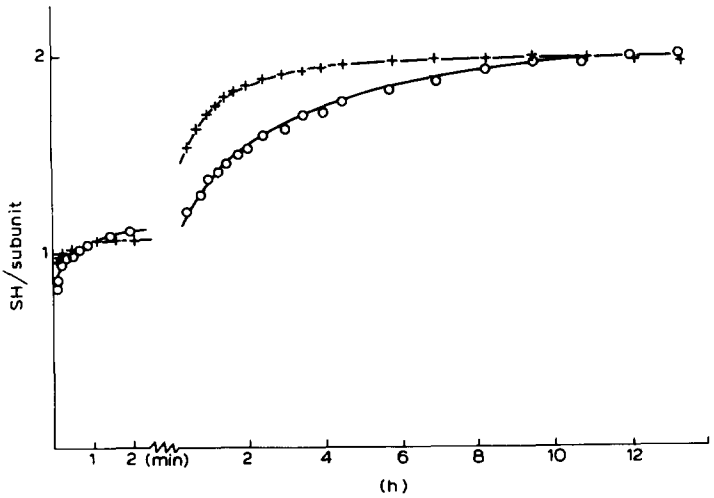

Fig. 1. Reaction of native and modified glyceraldehydephosphate dehydrogenase with DTNB. +, native glyceraldehydephosphate dehydrogenase; ○, HNB-glyceraldehydephosphate dehydrogenase.

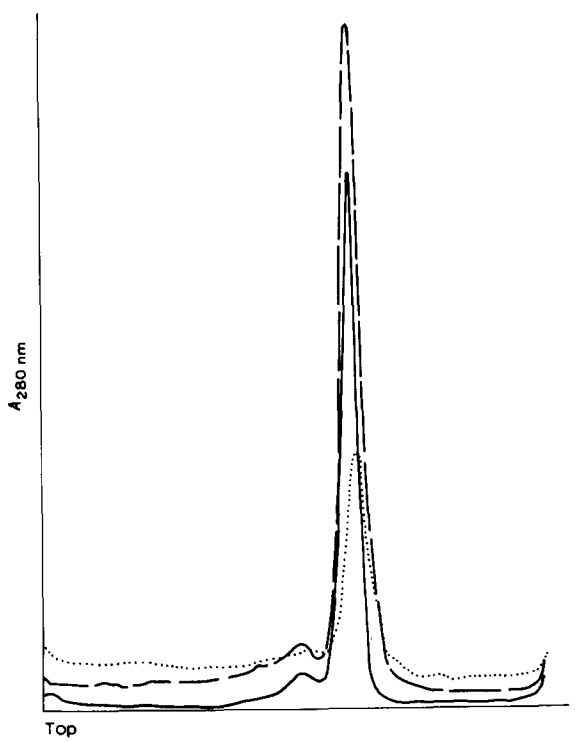

Fig. 2. Disc electrophoresis of native and HNB-glyceraldehydephosphate dehydrogenase. The gels had been scanned at 280 nm. Solid line, native glyceraldehydephosphate dehydrogenase; dotted line, HNB-glyceraldehydephosphate dehydrogenase; dashed line, mixture of both.

TABLE I

HNB-LABELED PEPTIDES ISOLATED FROM HYDROLYSATES OF HNB-GAPDH

Peptide	1	2*
Aspartic acid	11.7 nmol	4.0 nmol
Arginine	10.1 nmol	4.6 nmol
HNB groups	13.5 nmol	9.0 nmol

* Values corrected by background subtraction.

examined following hydrolysis by constant-boiling HCl for 16 h in sealed tubes.

In the fingerprint experiment, carried out with thoroughly digested HNB-glyceraldehydephosphate dehydrogenase, only one yellow spot was detected which was also ninhydrin-positive.

Active-site titration

Glyceraldehydephosphate dehydrogenase is also known to posses esterolytic activity [36—38]. The reactive SH group acts as an acyl acceptor during the formation of the acyl enzyme intermediate [39]. This reactive SH group is located at the active site for dehydrogenase activity since it is also involved in the formation of the acyl enzyme intermediate in this reaction.

The active site concentration has been estimated by following the liberation of *p*-nitrophenol from *p*-nitrophenylacetate [36—39]. In the experiment the initial burst was almost completed within 30 s after mixing. Shortly afterwards the increase of the absorbance at 400 nm became linear. With HNB-glyceraldehydephosphate dehydrogenase, the plot of the absorbance at 400 nm versus the reaction time gave a straight line from the beginning. This indicates that no acyl enzyme is formed, in contrast to the native enzyme. The slope was only slightly steeper than that of the control, in which the enzyme had been omitted. The increase of the hydrolysis rate might be due to the unspecific catalytic effect of groups at the surface of the protein molecule.

Proteolytic digestion

When the rate of the proteolysis by chymotrypsin was followed by automatic titration with 0.01 M NaOH under nitrogen (the samples were not previ-

TABLE II

DETERMINATION BY THE GEL FILTRATION TECHNIQUE OF THE COENZYME BINDING BY NATIVE AND MODIFIED GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE

	Glyceraldehyde-phosphate dehydrogenase	HNB-glyceraldehyde-phosphate dehydrogenase
Protein	195 nmol	120 nmol
NAD^+ ("negative peak")	185 nmol	8 nmol
NAD^+ bound per subunit	0.95	0.06

TABLE III

EQUILIBRIUM DIALYSIS EXPERIMENTS

Protein	NAD^+ in the outer dialysate* (μM)	Protein concn (μM)	Excess NAD^+ in the inner dialysate (μM)	NAD^+ molecules bound per glyceraldehyde-phosphate dehydrogenase subunit
Glyceraldehydephosphate dehydrogenase	34.2	268	38.3	0.14
HNB-glyceraldehydephosphate dehydrogenase	34.2	272	0	0
Glyceraldehydephosphate dehydrogenase	63.7	312	61.1	0.20
HNB-glyceraldehydephosphate dehydrogenase	63.7	274	0	0
Glyceraldehydephosphate dehydrogenase	223.4	277	82.9	0.30
HNB-glyceraldehydephosphate dehydrogenase	223.4	261	0	0

* Determined enzymatically.

ously denatured), no significant difference in the hydrolysis rate between the native and the HNB-glyceraldehydephosphate dehydrogenase appeared.

Binding of NAD^+ to the native and the modified apoenzyme

To test the binding ability for coenzyme of the native and the modified enzymes, the method described by Pfleiderer and Auricchio [40] was employed. The oxidized coenzyme bound to the apoenzyme was evaluated from the NAD^+ deficiency in the negative peak fractions. The results of this experiment (Table II) show that HNB-glyceraldehydephosphate dehydrogenase does not bind the coenzyme. In the equilibrium dialysis experiment, the same result was obtained (Table III). When the binding of NAD^+ to the apoenzyme was followed by recording the difference spectrum between 320 and 450 nm (the Racker band), the HNB-glyceraldehydephosphate dehydrogenase showed no such band in the wavelength range observed, whereas native glyceraldehydephosphate dehydrogenase developed the Racker band immediately after mixing.

Discussion

The investigation of the reaction of Koshland's reagents with yeast glyceraldehydephosphate dehydrogenase showed that tryptophan residues could be specifically modified. The SHNBB proved to be especially suitable due to its solubility in water and its comparatively high resistance to hydrolysis [41]. The modification converts the indole nucleus into a substituted 2,3-dihydroindole. This reaction cannot be reversed in the protein [32,41,42]. One particular difficulty arises from the fact that glyceraldehydephosphate dehydrogenase possesses one extremely reactive SH group per subunit [43,44]. Disulfide formation with DTNB proved to be the best way to achieve the necessary protection during the modification reaction. The protecting group can easily be removed, and the protected protein remains soluble. The chemical properties of the two SH groups of the subunit remain largely unchanged after the modification. The small difference of the reaction rate of the reactive SH group with

DTNB, as shown by the stopped-flow experiment, might be caused by steric hindrance exerted by the HNB group in the vicinity.

Modification of the glyceraldehydephosphate dehydrogenase with SHNBB causes a virtually complete loss of its enzymatic activity.

To estimate the number of HNB groups attached covalently to the protein one must take into account that varying amounts of the reagent or its hydrolysis products are incorporated into the protein, presumably into hydrophobic regions, without being covalently bound. The absorbance of this material is almost identical in the visible and the ultraviolet ranges with that of the tryptophan-attached HNB groups. To remove this non-covalently bound material, the protein had to be unfolded in urea solution.

To establish the site of the modification in the primary structure the labeled peptide was isolated after proteolytic digestion. Very long incubation times were required because amino acid sequences in the vicinity of the tryptophan residues hydrolyze very slowly [45].

Separation and amino acid analyses of the labeled peptides thus generated revealed two peptides with identical amino acid composition one of which contained a stoichiometric amount of HNB groups, the other one twice as much. The existence of the latter peptide can be explained by the observation of Tucker et al. [41] that reaction of tryptophan residues with SHNBB can yield different products depending on whether one or two HNB substituents are bonded to the tryptophan side chain.

The amino acid composition of these two peptides does not fit any other sequence around the three tryptophans of the subunit except that of Trp-193. In the lobster muscle enzyme this Trp-193 is close to Lys-183 (which has been found essential for enzymatic activity [46]) in space but is separated in the sequence by nine residues which form two antiparallel strands of a β-pleated sheet [47].

The coenzyme binding experiments show that formation of the holoenzyme from the apoenzyme has been inhibited in the case of the modified glyceraldehydephosphate dehydrogenase. It had to be confirmed that partial denaturation (transconformation) caused by the modification was not responsible for the inhibition of the coenzyme binding. Any statement concerning the conformation of a certain protein implies that the conclusions may hold true only for the methods employed. Thus we can state that no significant transconformation could be observed in the electrophoretic mobility at slightly basic pH, in the λ_c values computed from the ORD curves (unpublished results) according to Yang and Doty [48], from the reactivity of both the "reactive" and the "buried" SH groups, and from the hydrolysis rate by chymotrypsin.

The results obtained from all methods used agree in indicating that there is no binding of the NAD^+ to the apoenzyme in the modified enzyme species. Thus the statement seems well founded that indeed the modification of the tryptophan-193 abolishes the enzymatic activity of the glyceraldehydephosphate dehydrogenase.

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