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RESEARCH NOTE

THE INTERACTION OF ACRYLAMIDE WITH GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE. STRUCTURAL MODIFICATIONS IN THE ENZYME STUDIED BY FLUORESCENCE TECHNIQUES

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Abstract—The interaction of acrylamide with rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GPDH) has been investigated in Tris buffer, pH 7.5. When GPDH containing about 1 mol NAD per mol of tetramer is incubated with acrylamide (0.01–0.1 *M*), the tryptophan emission of GPDH, initially quenched by acrylamide, slowly increases to a value exceeding that recorded before the addition of acrylamide. This effect is not observed in apoenzyme solutions, indicating that the enhancement of fluorescence results from the dissociation of some NAD from the acrylamide treated GPDH. Acrylamide inactivates GPDH but 1 m*M* NAD protects the enzyme from inactivation. The addition of acrylamide to GPDH, labeled with fluorescein-5-isothiocyanate (GPDH-FITC) increases the fluorescence and decreases the polarization of fluorescein. The fluorescent sulfhydryl reagent N-iodoacetyl-N′-(5-sulfo-1-naphthyl)ethylenediamine induces similar changes in the fluorescence properties of GPDH-FITC. This reagent, however, fails to react with GPDH preincubated with acrylamide and the titration of acrylamide treated GPDH with the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) indicates the loss of up to 7 cysteine residues per tetramer. Acrylamide also decreases the heat stability of GPDH. Altogether, the data indicate that acrylamide covalently reacts with the active site cysteine residues of GPDH and subsequently induces a conformational change in the enzyme.

INTRODUCTION

The interactions of acrylamide, a neurotoxic agent, with various biological systems have been intensively investigated (Miller and Spencer, 1985). Acrylamide induces various morphological changes in nerve cells but the molecular mechanisms of its toxicity are not yet completely understood (Miller and Spencer, 1985). Although a recent report indicating the existence of strong interactions between acrylamide and human serum albumin, monellin and ovalbumin (Blatt *et al.*, 1986) has been disputed (Eftink and Ghiron, 1987), acrylamide is known to interact, usually as an inhibitor, with several enzymes. These include liver alcohol dehydrogenase (Woronick, 1961; Eftink and Selvidge, 1982), glucose 6-phosphate dehydrogenase (Harrison, 1974), acetylcholinesterase (Ngo, 1976), enolase and phosphofructokinase (Howland *et al.*, 1980), trypsin and chymotrypsin (Eftink and Ghiron, 1987) and transglutaminase (Signorini *et al.*, 1988). Acrylamide is also a commonly used quencher of protein fluorescence (Eftink and Ghiron, 1981). Therefore, a detailed understanding of the interactions of acrylamide with proteins is significant in relation to both the neurotoxicity of acrylamide and its use as a fluorescence quencher.

Acrylamide has also been shown to inhibit the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GPDH†, EC 1.2.1.12) both in purified form from rabbit muscle (Sabri, 1983) and in rat brain homogenates (Howland *et al.*, 1980; Sabri, 1983). But a detailed structural investigation of the interaction of acrylamide with GPDH has not been reported.

In this report we present various spectroscopic data indicating that acrylamide covalently binds to the cysteine residues of rabbit muscle GPDH and subsequently induces a conformational change in the enzyme. This conformational change appears to be responsible for the decreased heat stability of the enzyme in the presence of acrylamide.

MATERIALS AND METHODS

Unless otherwise indicated, all experiments were performed at 25°C in either 10 or 50 m*M* Tris buffer, pH 7.5, containing 1 m*M* EDTA. Rabbit muscle GPDH (Sigma Chemical Co., St Louis, MO) was stored at 4°C as a crystalline suspension in 2.6 *M* ammonium sulfate, pH 7.5, containing 1 m*M* EDTA and 4 m*M* 2-mercaptoethanol.

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†*Abbreviations*: ADP, adenosine 5′-diphosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein-5-isothiocyanate; GPDH, glyceraldehyde-3-phosphate dehydrogenase; I-AEDANS, N-iodacetyl-N′-(5-sulfo-1-naphthyl) ethylenediamine; NAD, β-nicotinamide adenine dinucleotide; Tris, tris(hydroxylmethyl)aminomethane.

Before each experiment an aliquot of the enzyme suspension was desalted using a Sephadex G-25 column and filtered through an either 0.2 or 0.45 μm disposable filter assembly (Gelman Sciences, Inc., Ann Arbor, MI). Unless otherwise indicated, the enzyme preparations used in the experiments contained about 0.6–2 mol of NAD per mol of tetramer, as determined from the ratio of the absorbances of GPDH at 280 and 260 nm (Fox and Dandliker, 1956). The molar extinction coefficient under these conditions, 1.3×10^5 M^{-1} cm^{-1} at 280 nm, was estimated from the data of Fox and Dandliker (1956) and used to determine the enzyme concentrations. The molecular weight employed for GPDH was 145 000. All enzyme concentrations are given in terms of tetramers. Protein concentrations of the solutions containing acrylamide were determined by using the coomassie blue binding assay (Bio-Rad Laboratories, Richmond, CA). GPDH activity was assayed at 25°C in 50 m*M* Tris buffer, pH 8.5, using the procedure of Scheek and Slater (1982).

ApoGPDH was prepared at room temperature by using a slightly modified version of the cation exchange method of Scheek and Slater (1982). A solution of GPDH, desalted with a 5 m*M* Tris buffer containing 1 m*M* EDTA (pH 6.4), was applied to a CM-Sephadex C-50 column (1 × 20 cm) and the column was washed with the same buffer until the absorbance at 260 nm decreased to negligible values. GPDH was then eluted with a 5 m*M* Tris buffer containing 1 m*M* EDTA and 0.1 *M* ammonium sulfate (pH 7.5). The enzyme solutions prepared in this manner had 280–260 absorbance ratios of 1.80–1.90, indicating the presence of < 0.3 mol NAD per mol of tetramer (Fox and Dandliker, 1956).

GPDH was covalently labeled by incubating a 6 μ*M* solution for 1 h at room temperature with a 10-fold excess of fluorescein-5-isothiocyanate (FITC, isomer 1 from Molecular Probes, Inc., Eugene, OR). At the end of the incubation period the unreacted dye was removed using a Sephadex G-25 column. The amount of FITC attached to the enzyme was determined from its absorbance at 492 nm using an extinction coefficient of 6.3×10^4 M^{-1} cm^{-1} and the concentration of the labeled enzyme was determined as before, after correcting for the FITC absorbance at 280 nm using an extinction coefficient of 2.2×10^4 M^{-1} cm^{-1}, calculated from the absorbance of free FITC. The ratio of the concentration of FITC label to the concentration of GPDH tetramer varied between 2.9–3.5. Labeled GPDH, which retained about 90% of its original specific activity, was used for the experiments on the same day it was prepared.

Fluorescence and light scattering data were obtained with a SPEX Fluorolog II spectrofluorometer. The polarization data were collected in the L-format configuration (Lakowicz, 1983). Excitation and emission wavelengths are given in the figure captions. Tryptophan emission spectra obtained in the presence of acrylamide by exciting at 290 nm were corrected for acrylamide absorption (Parker, 1968) using an extinction coefficient of 0.698 M^{-1} cm^{-1}. During the light scattering measurements the solutions were illuminated at 320 nm and the intensity of the scattered light at the same wavelength was recorded at 90° with respect to the direction of the incident light. Light scattering is expressed in arbitrary units as the ratio of the intensity of the scattered light to that of the incident light.

Gel filtration chromatography was performed at room temperature (21-23°C) with a 1 × 116 cm Sephadex G-200 column. Prior to chromatography, GPDH (6–8 μ*M*) was incubated without any additives for 75 min and either with acrylamide (0.06 *M*) for 90 min or with 5-fold excess of N-iodoacetyl-N′-(5-sulfo-1-naphthyl)ethylenediamine (I-AEDANS) for 15 min. Approximately 2 mg protein were loaded on the column during each run and eluted with 50 m*M* Tris buffer, pH 7.5, containing 1 m*M* EDTA. Absorbances of the 0.9 mℓ fractions were recorded at 280 nm. The column was calibrated with aldolase and lactic dehydrogenase from rabbit muscle and bovine serum albumin.

The number of sulfhydryl groups on GPDH were determined by using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) with an extinction coefficient of 1.36×10^4 M^{-1} cm^{-1} at 412 nm for the 2-nitro-5-thiobenzoate anion (Elman, 1959).

Acrylamide (Sigma Chemical Co., St Louis, MO) was dissolved in ethanol, filtered through a fine sintered glass filter to remove an insoluble residue and recrystallized under vacuum. A 4.0 *M* stock solution was prepared in water and stored frozen in the dark. During the experiments small aliquots (5–25 μℓ) from this solution were added to the GPDH solutions to obtain the desired final concentrations. I-AEDANS was from the Sigma Chemical Co. (St Louis, MO) and used without further purification.

RESULTS AND DISCUSSION

The addition of acrylamide in a concentration range commonly used for fluorescence quenching (0.01–0.1 *M*) to GPDH (0.4–0.7 μ*M*) rapidly decreases the fluorescence of GPDH, possibly as a result of the well known quenching of the fluorescence of tryptophan residues by this reagent (Eftink and Ghiron, 1981). As shown in Fig. 1, however, almost immediately the fluorescence begins to increase and levels off at a value exceeding that recorded before the addition of acrylamide. To quantify the enhancement of GPDH fluorescence without the quenching effect of acrylamide, one sample of GPDH was incubated with acrylamide (0.06 *M*) for 1 h and acrylamide was subsequently removed by desalting the solution. The fluorescence emission spectrum of this solution along with that of a control solution incubated without acrylamide are shown in Fig. 1 (inset). No shift in the position of the fluorescence maximum of GPDH (at approximately 345 nm) treated with acrylamide is observed.

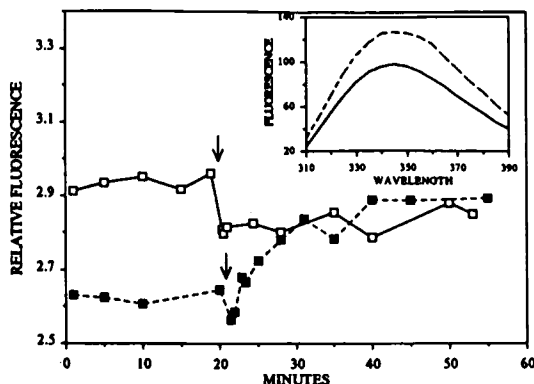

Figure 1. The effect of acrylamide (0.06 *M*) on the fluorescence of 0.8 μ*M* apoGPDH with (■) or without (□) 0.9 μ*M* NAD in 50 m*M* Tris buffer. Arrows indicate when acrylamide was added to the enzyme solutions. Excitation and emission wavelengths were 290 and 345 nm, respectively. Inset: emission spectra of GPDH incubated with (- - -) or without (—) acrylamide for 60 min. Prior to the measurements both solutions were desalted and the absorbances were adjusted to the same value at the excitation wavelength of 290 nm.

When acrylamide is added to an apoGPDH solution, the fluorescence of the enzyme is quenched but no subsequent increase in the fluorescence is observed (Fig. 1). Because the binding of NAD to apoGPDH decreases the tryptophan fluorescence of the enzyme (Velick, 1958; Scheek *et al.*, 1979), the increase in fluorescence observed during the reaction of acrylamide with GPDH containing NAD (Fig. 1) probably reflects the dissociation of some NAD into the solution as a result of a decreased affinity between GPDH and NAD.

To investigate the effect of acrylamide on tryptophan absorption, the absorption spectrum of a 0.8 μM GPDH solution was recorded following its incubation with 4 mM acrylamide for 90 min at 25°C. To correct for the contribution of acrylamide absorption, the blank also contained 4 mM acrylamide. The spectrum obtained for GPDH in this manner in the region 260–340 nm was identical to the absorption spectrum of a GPDH solution incubated without acrylamide.

In the presence of acrylamide GPDH rapidly loses its activity. During the experiment shown in Fig. 2, 0.4 μM GPDH incubated with 8 mM acrylamide at 25°C lost about 82% of its original activity in 26 min. In a similar experiment, two solutions of GPDH were incubated with 0.02 and 0.04 M acrylamide, respectively, for 2 h. At the end of the incubation period no activity could be detected in either solution, whereas a control solution incubated without acrylamide was still active. Sabri (1983) has also reported that 5 mM acrylamide completely inactivates micromolar concentrations of GPDH in 90 min.

Within the time period of the experiment shown in Fig. 2, 1 mM NAD completely prevents the inactivation of GPDH by acrylamide, whereas 1 mM ADP slows down the inactivation process. Muronetz *et al.* (1976) have shown that the incubation of rat muscle GPDH with 10 mM ADP at 25°C in 0.1 M phosphate buffer, pH 7.3, results in a loss of about 50% of the GPDH activity. Under the conditions of our experiments, however, ADP slightly increases the activity of rabbit muscle GPDH (Fig. 2).

We investigated the kinetics of the interaction of acrylamide with GPDH by following the changes induced in the fluorescence of fluorescein covalently attached to GPDH. Acrylamide does not quench fluorescein fluorescence (Eftink *et al.*, 1987), so the analysis of the data was not complicated by an initial drop in fluorescence, as was observed in the case of tryptophan fluorescence (Fig. 1).

Dilution of GPDH labeled with FITC (GPDH-FITC) with buffer to a final concentration of 0.4 μM results in a slight increase in FITC fluorescence lasting about 10 min, after which a much slower increase in fluorescence continues for several hours [Fig. 3(A)]. This change in fluorescence, accompanied by a slow decrease in the polarization of FITC [Fig. 3(B)], probably reflects the gradual dissociation of tetrameric GPDH into smaller units. If acrylamide is added to a solution of GPDH-FITC already incubated in buffer for 10 min, a large increase in the fluorescence of FITC is observed [Fig. 3(A)]. Depending on the concentration of acrylamide, the fluorescence continues to increase for 1–3 h. During the same period the polarization of FITC decreases from an initial value of about 0.2 to about 0.11 [Fig. 3(B)]. Despite these changes in

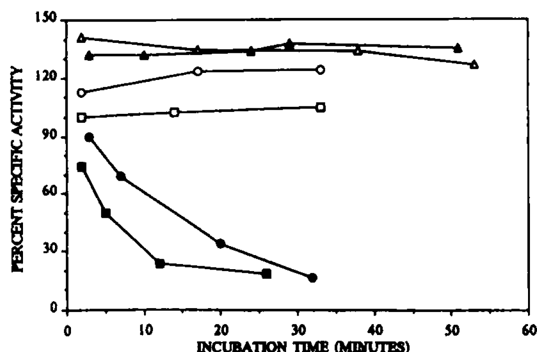

Figure 2. Specific activity (μmol substrate reacted per minute per mg GPDH) of 0.4 μM GPDH incubated with (solid symbols) or without (open symbols) 8 mM acrylamide in the presence of no other additives (□,■) and either with 1 mM NAD (△,▲) or ADP (○,●) in 10 mM Tris buffer. Specific activity is expressed as the percent of the initial activity of GPDH incubated without any additives.

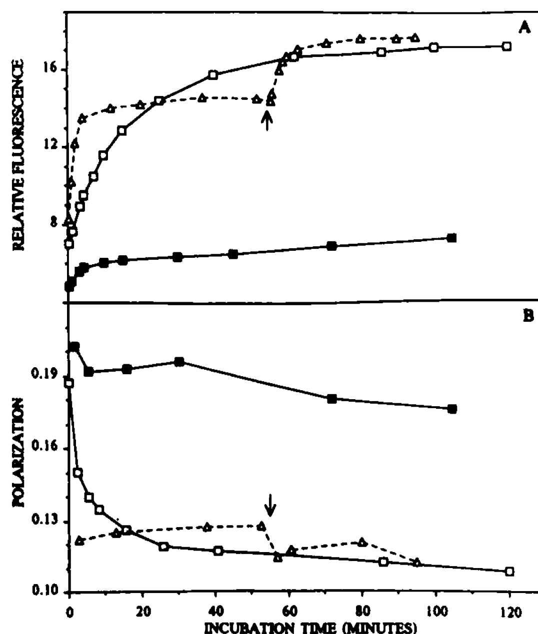

Figure 3. The effect of acrylamide on the emission (A) and polarization (B) of 0.4 μM GPDH-FITC. GPDH-FITC was incubated either without any additives (■) or with 0.08 M acrylamide in the absence (□) or presence of 1 mM ADP (△) in 10 mM Tris buffer. Arrows indicate when acrylamide was added to the ADP containing solution. Excitation and emission wavelengths were 490 and 517 nm, respectively.

fluorescence and polarization values, no spectral shifts were observed in the emission spectrum of FITC.

When GPDH-FITC is diluted with a buffer containing 1 mM ADP, FITC fluorescence rapidly increases, while the polarization decreases to a value of about 0.12 within 2 min (Fig. 3). Dilution with 1 mM NAD also causes similar changes in the fluorescence and polarization of FITC (data not shown). When acrylamide is subsequently added to a solution of GPDH-FITC incubated with ADP, both the fluorescence and polarization of FITC change further and eventually reach the same final values as those obtained in the absence of ADP (Fig. 3). These results, in agreement with the failure of ADP to protect GPDH against inactivation (Fig. 2), indicate that in the presence of acrylamide the enzyme attains the same final form whether or not ADP is present. The addition of acrylamide to a solution of GPDH-FITC incubated with NAD, however, causes very small changes in the fluorescence and polarization of FITC. For this reason, we performed no further experiments with this system.

The changes in FITC fluorescence observed after the addition of acrylamide to GPDH-FITC incubated with or without ADP were found to obey a pseudo first order relationship described by Eq. 1.

$$\ell n(F_e - F_t) = \ell n(F_e - F_i) - k'(t) \quad (1)$$

In this equation t is time in minutes and k' is the pseudo first order rate constant equal to the product of the second order rate constant and the total acrylamide concentration which is assumed to stay constant during the reaction. F_i and F_e are the initial and the equilibrium values of fluorescence, respectively, and F_t is the fluorescence at a given time during the reaction.

In the GPDH concentration range 0.1–0.9 μM, the pseudo first order rate constants obtained from Eq. 1 were independent of GPDH concentrations. The rate constants depend on acrylamide concentrations, but, as shown in Fig. 4, the lines obtained by plotting the values of k' against the acrylamide concentrations do not extrapolate to the origin at zero acrylamide concentration, as would be expected from a true pseudo first order reaction. The values of the slopes and intercepts of the lines in Fig. 4, obtained through a least squares analysis, were 0.31 M^{-1} min^{-1} and 0.029 min^{-1}, respectively, for the bottom line (without ADP) and 1.86 M^{-1} min^{-1} and 0.088 min^{-1}, respectively, for the top line (with ADP).

A plot of the kind shown in Fig. 4 implies an equilibrium between acrylamide and GPDH-FITC, with the slope and the intercept corresponding to the forward and reverse rate constants, respectively (Frost and Pearson, 1961). In this context we note that at least one enzyme, enolase, is inactivated reversibly by acrylamide (Howland *et al.*, 1980). The possibility of an equilibrium reaction between GPDH-FITC and acrylamide is, however, discounted on two grounds. First, as indicated earlier, after a long enough incubation period, regardless of the concentration of acrylamide (in the range 4–100 mM), GPDH is totally inactivated. If an equilibrium existed at lower acrylamide concentrations some fraction of the enzyme would be expected to remain active. Furthermore, when acrylamide is removed from GPDH during gel filtration using a Sephadex G-200 column, the enzyme remains inactive. Second, if an equilibrium existed between GPDH-FITC and acrylamide, the addition of more acrylamide to a mixture of GPDH-FITC and acrylamide after the fluorescence of fluorescein had stopped changing would be expected to cause a further increase in the fluorescence as the equilibrium shifted towards the formation of more altered GPDH. To test this possibility, 0.4 μM GPDH-FITC was incubated with 0.02 M acrylamide for 145 min. At the end of the incubation period, fluorescence and polarization values of the solution were recorded and more acrylamide was added in two steps to bring the final concentration first to 0.04 M and then to 0.08 M. No changes in the fluorescence or polarization values of the solution were observed, indicating that no measurable amount of unreacted GPDH-FITC was present. These considerations and the results given in Fig. 4 indicate that although the kinetic data appear to be pseudo first order, the actual mechanism of the interaction of acrylamide with GPDH is possibly more complex.

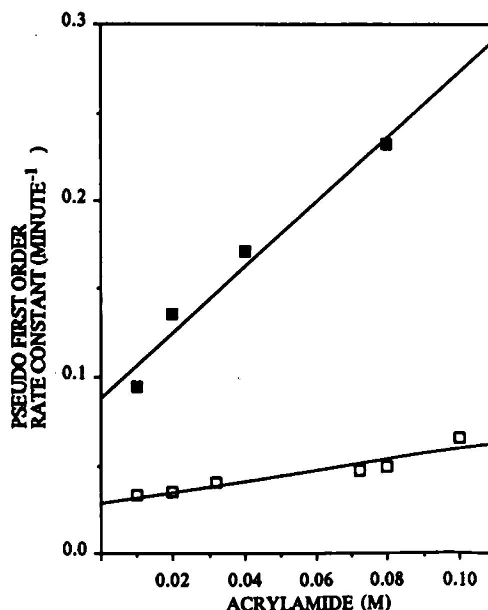

Figure 4. The dependence of the pseudo first order rate constants for the interaction of acrylamide with GPDH-FITC to acrylamide concentrations with (■) or without (□) 1 mM ADP.

We have also investigated the interaction of I-AEDANS with GPDH under various conditions. I-AEDANS covalently binds to the active site cys-149 residues of GPDH resulting in the inactivation

of the enzyme (Henis *et al.*, 1979). ApoGPDH is covalently labeled with a stoichiometry of 4 mol I-AEDANS per mol of GPDH and a large increase in the fluorescence of bound I-AEDANS, compared to that of free dye, is observed (Henis *et al.*, 1979).

To check the effect of acrylamide on the interaction of I-AEDANS with GPDH, two 3 μ*M* GPDH solutions one of which contained 0.04 *M* acrylamide were incubated for 30 min. At the end of the incubation period both solutions were diluted 10-fold with buffer and I-AEDANS was added to each one at a final concentration of 4.6 μ*M*. As indicated by the fluorescence increase at 500 nm, I-AEDANS rapidly reacted with GPDH [Fig. 5(A)]. In the case of GPDH treated with acrylamide, however, only a very slight increase in fluorescence was observed [Fig. 5(A)]. A control experiment indicated that preincubation of I-AEDANS with 0.01 *M* acrylamide for 10 min had no effect on its subsequent reactivity towards GPDH.

In another experiment, a 0.4 μ*M* solution of GPDH-FITC was incubated with or without 0.02 *M* acrylamide at 25°C for about 3 h. At the end of the incubation period a small aliquot from an I-AEDANS stock was added to both solutions to give a final concentration of 4.5 μ*M* and the fluorescence and polarization values of the fluorescein moiety of GPDH were monitored. As shown in Fig. 5(B), after the addition of I-AEDANS to the GPDH-FITC solution incubated without acrylamide, the fluorescence of fluorescein increased while its

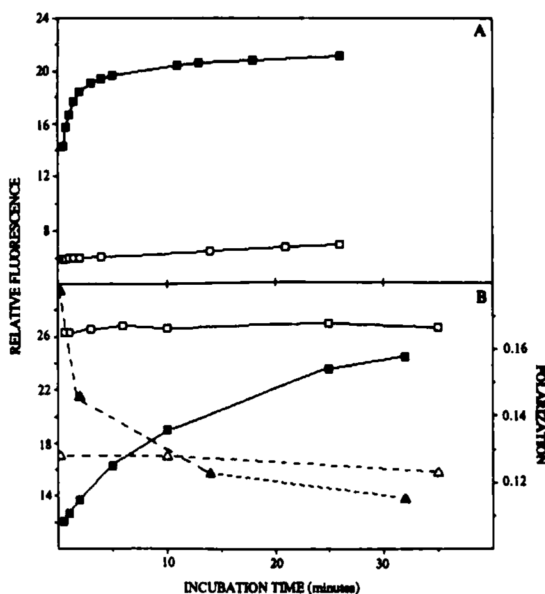

Figure 5. (A) The change in fluorescence at 500 nm during the reaction of I-AEDANS (4.6 μ*M*) with GPDH (0.3 μ*M*) incubated with (□) or without (■) acrylamide (0.04 *M*). Excitation wavelength was 380 nm. (B) The effect of I-AEDANS (4.5 μ*M*) on the fluorescence (left axis, □,■) and polarization (right axis, △,▲) of GPDH-FITC (0.4 μ*M*) incubated with (□,△) or without (■,▲) acrylamide (0.02 *M*). Excitation and emission wavelengths were 490 and 517 nm, respectively. The experiments were performed in 10 m*M* Tris buffer.

polarization decreased, but no changes in the fluorescence and polarization values of the solution containing acrylamide were observed (Fig. 5(B)].

Henis *et al.* (1979) have shown that apoGPDH labeled with I-AEDANS binds NAD with decreased affinity. As discussed earlier, our data presented in Figs. 1 and 2 indicate that acrylamide also decreases the affinity of GPDH towards NAD and high concentrations of NAD prevents the reaction of acrylamide with GPDH. Likewise, the addition of I-AEDANS to GPDH in the presence of 1 m*M* NAD, unlike the results given in Fig. 5(A), results in a very small increase in the fluorescence of I-AEDANS (data not shown). Since acrylamide and I-AEDANS also induce similar changes in the fluorescence properties of GPDH-FITC (Figs. 3 and 5), the most plausible explanation for our data is that, similar to the reaction of I-AEDANS with GPDH, acrylamide also covalently binds to the cys-149 residues located in the active sites and consequently inactivates the enzyme.

To complement this conclusion, we determined the changes in the number of cysteine residues of GPDH following its interaction with acrylamide using DTNB. A 2 μ*M* solution of GPDH incubated at 25°C for 10 min had 4 and 10.5 cysteine residues per tetramer, determined 0.5 and 40 min after the addition of an excess amount of DTNB, respectively. The incubation of the enzyme for 60 min before the addition of DTNB did not change the number of reactive cysteine residues. When the enzyme was incubated with 0.04 *M* acrylamide at 25°C for 10 min before the addition of DTNB, however, the number of reactive cysteine residues per tetramer dropped down to approximately 1.9 and 6, determined, as before, 0.5 and 40 min after the addition of DTNB, respectively. These values were further decreased to approximately 1.5 and 3.5 after the enzyme was incubated with acrylamide for 60 min. These results indicate that acrylamide reacts with about 5 cysteine residues per tetramer in 10 min and that longer incubation periods result in the reaction of up to 7 cysteine residues with acrylamide.

The large changes observed in the fluorescence and polarization values of GPDH-FITC during incubation with acrylamide and I-AEDANS may arise either from an increase in the degree of dissociation of tetrameric GPDH or from a conformational change in the enzyme, both induced by the alkylation of the cys-149 residues.

To check for the effects of acrylamide and I-AEDANS on the oligomeric structure of GPDH, gel filtration experiments were performed using a Sephadex G-200 column as explained in the Materials and Methods section. The elution profiles obtained for GPDH incubated without any additives and with either acrylamide or I-AEDANS were similar. The molecular weight estimated for GPDH from the results of the gel filtration experiments was about 1.1×10^5, indicating that the unaltered

enzyme itself was slightly dissociated under the conditions of our experiments.

Since the results of the gel filtration experiments rule out the additional dissociation of tetrameric GPDH in the presence of acrylamide and I-AEDANS, to explain the spectroscopic data we propose that the interactions of these reagents with the cysteine residues of GPDH induce a conformational change in the enzyme. This argument implies that the formation of a covalent bond between the cys-149 residues of GPDH and its substrate glyceraldehyde 3-phosphate (Harris and Waters, 1976) may also introduce a conformational change in GPDH, detectable by the changes in various spectral properties of the tryptophan residues. This conclusion is supported by a recent report of perturbations observed in the UV resonance raman spectrum of the tryptophan residues of GPDH in the acyl enzyme intermediate formed during the reaction of the enzyme with glyceraldehyde 3-phosphate (Austin *et al.*, 1989).

It should, however, be noted that the kinetic analysis is complicated by the observation that up to 7 acrylamide molecules react, possibly each with a different rate constant, with each GPDH tetramer. Therefore, at this point the physical significance of the slope and intercept values obtained in Fig. 4 remains unclear.

During the preparations of enzyme solutions and gel filtration experiments, we repeatedly obtained smaller yields for the acrylamide treated GPDH samples eluted through the Sephadex G-25 or G-200 columns, compared to the yields of GPDH solutions incubated without acrylamide. These experiments were conducted at room temperature and an analysis of the data indicated that the protein losses, ranging from 5 to 15%, depend on the variations in the room temperature. The aggregation and subsequently, the precipitation of GPDH modified by acrylamide inside a Sephadex column can result in the loss of protein, so we investigated the temperature dependent aggregation of GPDH in the presence of acrylamide. The incubation of GPDH (1.5 μM) with acrylamide (0.08 M) at 28°C causes a large increase in the light scattering intensity of the solution over a 2 h period compared to that of the enzyme solution incubated without acrylamide (Fig. 6). The light scattering intensities of the enzyme solutions incubated with or without acrylamide at 20°C were, however, virtually identical (Fig. 6). The scattering intensity of a blank solution containing acrylamide but no enzyme was about 20-fold less than those of the GPDH solutions and remained constant during the incubation period. To quantify the loss of protein through aggregation, GPDH solutions (2 μM) were incubated in the dark either without any additives or with acrylamide (0.08 M) in water baths at either 21.5 or 30°C for 110 min. At the end of the incubation period each solution was filtered through a 0.2 μm filter and the protein concentrations of the filtrates were

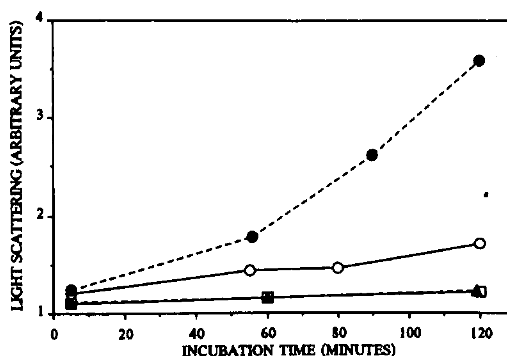

Figure 6. The light scattering intensity at 320 nm of GPDH (1.5 μM) solutions incubated with (solid symbols) or without (open symbols) acrylamide (0.08 M) at 20° (□,▲) and 28°C (○,●) in 50 mM Tris buffer.

determined. As indicated in Table 1, while the incubation of GPDH with acrylamide at 21.5°C resulted in the loss of only about 6% protein, the incubation of the enzyme with acrylamide at 30°C resulted in the retention of about 42% of the protein by the 0.2 μm filter. The decreased heat stability of GPDH appears to be another indication of a change in the conformation of the protein induced by the reaction of acrylamide with the cysteine groups. As expected, the presence of excess NAD in the incubation medium prevents the aggregation of GPDH by acrylamide (Table 1).

One of the most abundant enzymes in many tissues, GPDH, is known to interact with actin filaments and other cytoskeletal structures (Humphreys *et al.*, 1986). It has been suggested that in intact cells, in addition to its catalytic function in the glycolysis, this enzyme may also have a structural function (Humphreys *et al.*, 1986). On the other hand, acrylamide has been reported to disrupt at least one cytoskeletal structure, the vimentin intermediate filament network, in cultured cells (Eckert, 1985). Therefore, it is possible that the covalent

Table 1. Temperature dependent effect of acrylamide on the protein concentrations of GPDH solutions filtered through 0.2 μm filters at the end of an incubation period of 110 min*

Additive	Protein concentration (mg/mℓ)	
	21.5°C	30°C
None	0.274 ± 0.012	0.269 ± 0.010
Acrylamide	0.258 ± 0.012	0.157 ± 0.011
Acrylamide + NAD	0.275 ± 0.005	0.270 ± 0.005

*Experiment was performed in 50 mM Tris buffer, pH 7.5. Following concentrations were used: GPDH, 2 μM; acrylamide, 0.08 M; NAD, 1 mM. The values listed are the averages of the protein concentrations of duplicate solutions and the standard deviations. Visible turbidity developed at the end of the incubation period only in the solutions incubated at 30°C with acrylamide but without NAD.

modification of GPDH by acrylamide and the subsequent alterations induced in the conformation of the enzyme may interfere with the binding of this enzyme to the cytoskeleton and consequently, result in the development of some of the morphological changes observed in nerve cells during acrylamide intoxication.

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