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Sodium Perchlorate-Induced Inactivation of Rabbit Muscle Enolase: Partial Inactivation of Dimeric Enzyme and Dissociation into Inactive Monomers

Adel A. Al-Ghanim

A Thesis
in
The Department
of
Chemistry and Biochemistry

Presented in Partial Fulfilment of the Requirements
for the Degree of Master of Science at
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Montreal, Quebec, Canada

February 1994

Adel A. Al-Ghanim, 1994

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ABSTRACT

Sodium Perchlorate-Induced Inactivation of Rabbit Muscle Enolase: Partial Inactivation of Dimeric Enzyme and Dissociation into Inactive Monomers

Adel A. Al-Ghanim

Sodium perchlorate ($NaClO_4$) inactivates and dissociates enolase. In order to determine the relationship between these processes, the effects of $NaClO_4$ and NaCl on the activity and quaternary structure of rabbit muscle enolase were studied. To monitor dissociation, the enzyme was crosslinked with glutaraldehyde and then analyzed by SDS-PAGE. Enolase was labelled with dansyl chloride as a probe of conformational change.

Exposure of the labelled enolase to $NaClO_4$ resulted in a dramatic increase in the fluorescence intensity of dansyl-$\beta\beta$. Inactivation, dissociation and fluorescence intensity are time and [$NaClO_4$] dependent; NaCl has no effect on these processes. A comparison of inactivation and dissociation as a function of time and [$NaClO_4$], indicates that inactivation occurs prior to dissociation. Indeed, $NaClO_4$ inactivates the dimeric enzyme and causes dissociation. The increase in the fluorescence of dansyl-$\beta\beta$ was found to exactly follow neither inactivation nor dissociation.

This conclusion was confirmed by the occurrence of inactivation when dissociation was prevented. Dissociation could be prevented by covalently crosslinking the enzyme with bis(sulfosuccinimidyl) suberate or by the addition of substrate. In experiments using low concentrations of $NaClO_4$ (up to 0.4M), both treatments greatly decreased the rate of inactivation and the change in fluorescence

intensity; at high concentrations (> 1.0M), crosslinking provided almost no protection against inactivation. Conformational changes within the enzyme occurred as a result of exposure to $NaClO_4$, confirmed by the change in fluorescence intensity of tryptophan, dansyl-$\beta\beta$ and inactivation.

The fact that inactivation precedes dissociation and can occur in the absence of dissociation means that the observed effects of $NaClO_4$ on the activity of rabbit muscle enolase are due to a partial inactivation of dimeric enzyme and to a dissociation into inactive monomers.

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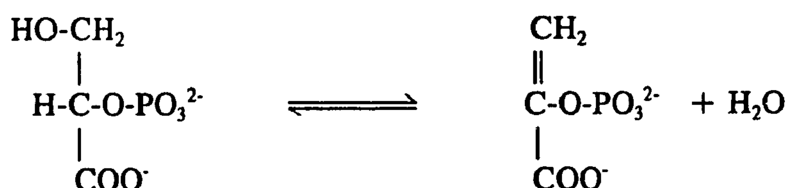
A. INTRODUCTION

Enolase (2-phospho-D-glycerate-hydrolase, E.C.4.2.1.11) catalyzes the reversible dehydration of 2-phosphoglycerate (PGA) to form phosphoenolpyruvate (PEP) with an absolute requirement for divalent cations such as Mg^{2+} which happens to give the highest activity (1).



Enolases mostly are dimeric enzymes with subunit molecular weights of 40 to 50 Kd (1,2). Mammalian tissues have three genes which code for enolase, their products are the α, γ and β subunits. Both homo- and hetero-dimers are formed with five of the six possible dimers reported to exist in vivo (3,4). These isozymes have very similar amino acid sequences but differ in sensitivity to inactivating agents such as pressure or chaotropic salts (5,6,7,8).

Recently, the 3-D structure of yeast enolase was determined (9,10,11). The molecule is a dimer of identical subunits, each composed of two domains. The main domain is an 8-fold α and β barrel. The active site is located in a deep cavity at the carboxylic end of the β-barrel. The smaller N-terminal domain has an α and β structure based on a three-stranded antiparallel meander and four helices (Fig. 1).

Most of the contacts at the dimer interface are between the side chains of the last helix (H) of the barrel domain in one subunit and the atoms of the main chain of the meander (strand 9 and 10) in the second subunit. There are three forces involved in the interactions between the subunits of the dimer: hydrophobic contacts, electrostatic attraction between two pairs of ions and finally hydrogen bonds. There is a deep cleft between the subunits which is accessible to the solvent.

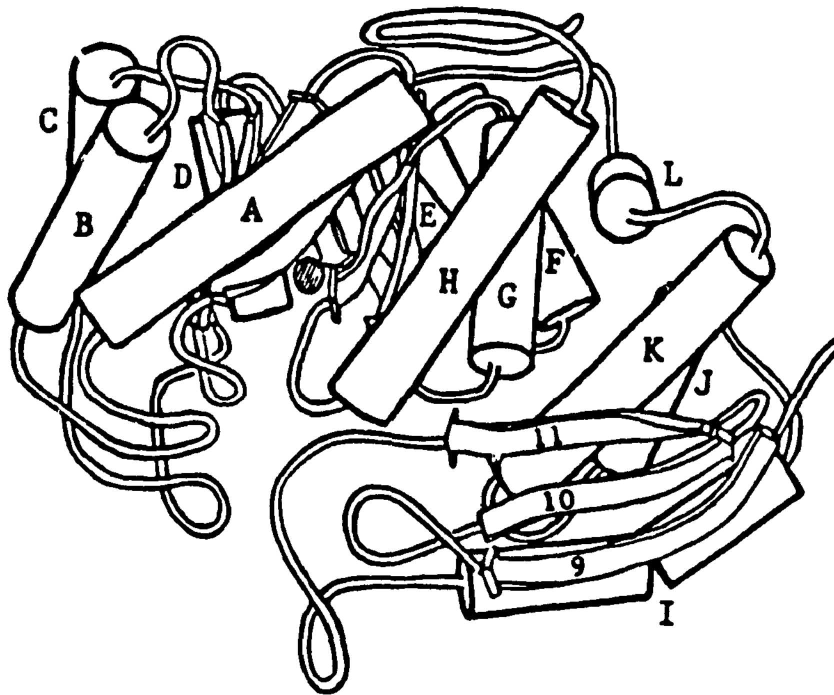
The study of the nature of the interactions that maintain the quaternary structure of enzymes and their influence on catalysis is essential for the understanding of cellular functions at the molecular level.

This work is focused on the rabbit muscle enolase isozyme. While rabbit muscle enolase was purified in 1961 (12), the primary structure was determined by protein sequencing in 1990 with molecular weight of 94 Kd (13).

The study of the oligomeric enzyme's inactivation and dissociation relationships is a necessary step towards understanding the subunit interactions and the catalytic activity. Thus, one must study the properties of the single subunits and compare them with those of the oligomer. Generating monomers is a critical process in this regard. Ideally, one should be able to observe the activity and the structure of the oligomeric enzyme without disrupting the tertiary structure.

However, oligomeric enzymes generally dissociate into subunits, usually accompanied by inactivation, under the action of various chemical or physical agents such as salts and denaturants or pressure and temperature (5,6,14,15). In some cases, dissociation occurs upon lowering the protein concentration.

Figure 1: A cartoon representation of an enolase subunit. It is organized into two domains [taken from Lebioda, L., Stec, B. and Brewer, J. M. (9)].



Three models could illustrate the inactivation process·

These differences depend on the forces present in the quaternary and tertiary structures. In the first model, the dimer is altered under the influence of chemical or physical inactivating agents and dissociates into inactive monomers. In the second model, the inactivation comprises one step, and the structure of the monomer is altered as a result of dissociation. In the third model, inactivation is a two-step process, occurring via the production of active monomers, followed by conversion into inactive monomers.

Whether dissociation of oligomeric enzymes induces active or inactive monomers has been extensively studied. Porter and Cardenas (16) have applied the techniques for immobilizing enzyme subunits in order to determine the effect of subunit interaction on the catalytic activity of pyruvate kinase isozymes. The immobilized enzyme was subjected to dissociation with guanidine hydrochloride, followed by removal of the free subunits and renaturation of the immobilized subunits. It was found that there is no enzymatic activity on the subunits which remained bound to the resin. They concluded that single immobilized subunits of bovine pyruvate kinase were inactive.

Another possibility that could account for the lack of enzymatic activity of immobilized monomers of pyruvate kinase is incorrect refolding of the immobilized subunits as they interact with the matrix.

Briganti et al., in 1989 (17), showed that freezing human alcohol dehydrogenase in liquid nitrogen or in dry ice/acetone, thawing at 20°C, and subsequent storage at 0°C produces stable monomers. Ehrig et al., in 1993 (18), concluded that the enzymatic activity of human alcohol dehydrogenase monomers implies that the intersubunit contacts of $\beta_1\beta_1$ isozymes are not crucial in establishing a catalytically competent enzyme. They employed the same freeze-thaw method, with thawing at 0°C instead of the conventional 25°C in order to prevent unwanted reassociation in this phase. Although the dimeric enzyme has optimal activity at low substrate concentration, the monomer has its highest activity at high substrate concentration. The differences in specific activity and Km between monomer and dimer suggests that dimerization may serve to modulate the catalytic properties.

These examples of dissociation of oligomeric enzymes illustrate that results obtained depend on the techniques used and/or the critical interactions of the subunits.

Yeast enolase has been extensively studied by a number of groups with regard to the possible activity or inactivity of the yeast enolase monomers. The first group used high salt concentration to induce dissociation. In 1968, Brewer and Weber (19), showed that incubation of 1mg/ml yeast enolase in 1M KCl and excess EDTA at 20 °C results in loss of activity and dissociation into inactive monomers. The monomers

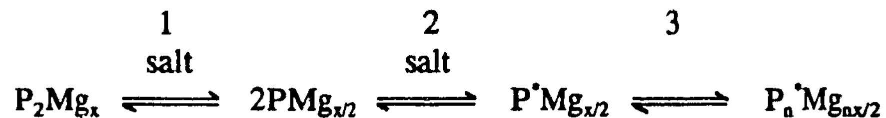
were detected by sedimentation equilibrium. They concluded that yeast enolase consists of two inactive subunits. This observation was consistent with Gawronski and Westhead's report (20). They used three techniques to confirm that yeast enolase dissociates in 1M KCl and KBr: subunit scrambling (formation of hybrids of native and acetylated enzyme), sedimentation equilibrium and gel filtration. The dissociated monomers were found to be inactive. It was concluded that the dimeric structure is essential for catalytic activity. This indicated that inactivation proceeds by model [2].

The second group used low enzyme concentration and temperatures of 40°C or 43°C. In 1971, Nagy (21) and Orman used the gel filtration technique to determine the molecular weight of yeast enolase in dimeric and monomeric forms. It was found that yeast enolase at a concentration of 0.7 μg/ml and a temperature of 43 °C exists as a monomer (even in the presence of substrate and Mg^{2+}) and is enzymatically active. Later Holleman, used similar conditions to dissociate yeast enolase (22). Dissociation and activity of the dimer were measured by the enzyme sedimentation technique, whereby the sedimentation of a band of enolase through its reaction mixture is followed by observing the appearance of the product phosphoenolpyruvate. The author also showed that the dissociated monomer is fully active.

It could therefore be concluded that the monomer of yeast enolase is active. This would imply that Brewer and Gawronski's results (the first group) can be

attributed to disruption of the tertiary structure by the salts used for dissociation, a process resulting in inactive monomer (model 3).

A similar picture has emerged from data on the dissociation of the mammalian enolases. In a sedimentation coefficient study, Winstead and Wold, 1965 (23), reported that the monomer of rabbit muscle enolase (the $\beta\beta$ isozyme) is devoid of enzymatic activity when the dimeric enzyme is incubated with EDTA and ammonium sulfate or in the presence of a high concentration (2M) of ammonium sulfate alone. The rate of dissociation is determined simply by following the rate of activity loss. The authors proposed the following model for the dissociation in 2M ammonium sulfate:



$$P_2Mg_x \overset{\text{1, salt}}{\rightleftharpoons} 2PMg_{x/2} \overset{\text{2, salt}}{\rightleftharpoons} P^*Mg_{x/2} \overset{3}{\rightleftharpoons} P_n^*Mg_{nx/2}$$

P_2Mg_x is native enolase, $PMg_{x/2}$ is a compact monomer, $P^*Mg_{x/2}$ is hypothetical unfolded or solvated monomer and $P_n^*Mg_{nx/2}$ is an aggregate of this modified monomer. According to this model, ammonium sulfate alone causes dissociation [reaction (1)] and a conformational change [reaction (2)]. It is postulated that P_2Mg_x is the only enzymatically active form in this model. In this study, however, one cannot tell if the activity loss is due to inactivation of the dimer (model [1]) since the authors didn't prove that the rate of activity loss represented dissociation.

In another study, Chorazyczeswki et al. (1987) (24) showed that immobilization of the native dimeric form of pig muscle enolase on Sepharose 4B activated with cyanogen bromide, followed by treatment with KBr, results in an

inactive monomer bound to the matrix. Consequently, it was concluded that the dimeric structure is essential for catalytic activity.

In both Winstead and Chorazyczewski's studies it is not clear whether inactivation is due to damage of the monomer's tertiary structure (caused by high concentration of salt or the immobilization) or to dimer inactivation and dissociation.

Mammalian brain enolase ($\alpha\alpha$, $\alpha\gamma$, $\gamma\gamma$) inactivation and dissociation relationships have also been extensively studied. In 1978, Marangos et al. (8) demonstrated that inactivation of mammalian brain enolase in 1M KCl or KBr is accompanied by dissociation. The subunit scrambling technique (the formation of $\alpha\gamma$ from a mixture of $\alpha\alpha$ and $\gamma\gamma$ during the reactivation) was used in this study. It was concluded that brain enolases have different stabilities during inactivation and that monomers are inactive. However, these authors did not determine the rates of dissociation.

In 1982, Kornblatt et al. (25) performed a pressure (instead of salt) induced reversible inactivation of brain enolases. Later, in 1987, Kornblatt obtained evidence of the quaternary structure of $\alpha\alpha$, $\alpha\gamma$ and $\gamma\gamma$ enolases under pressure, using subunit scrambling and glutaraldehyde crosslinking methods (5). It was found that inactivation was irreversible or partially reversible at a slow rate. Since subunit scrambling could occur at equilibrium, and the experiments were time consuming and laborious, it could not be determined whether the dissociation was complete and whether it occurred under the specific pressure chosen. However, it was concluded that the pressure-induced inactivation of enolase is a two-step process; the pressure

induces the dissociation of the enzyme followed by further changes in the resulting monomers.

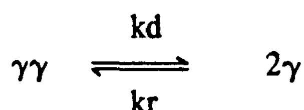
The glutaraldehyde crosslinking method (covalent linking of the monomers by the use of bifunctional reagent, glutaraldehyde) and subsequent analysis by SDS-polyacrylamide gel electrophoresis, was introduced by Jaenicke's group in their studies of lactate dehydrogenase (26). Kornblatt (5) and Trepanier (27) used this technique to qualitatively determine the relative proportion of dimers and monomers. On the other hand, crosslinking with glutaraldehyde allows the detection and quantitative evaluation of monomeric association and dimeric dissociation (26).

Kwao, in 1985 (28) immobilized the $\alpha\alpha$ isozyme of rabbit brain enolase in solid matrix. The immobilized enzyme was dissociated by 1M KBr; the salt and free subunits were removed. The relative activity of the immobilized monomers was 92% of the immobilized dimer's. However, the immobilized dimer had 74% activity of the native enolase. Kwao concluded that the immobilized monomer of enolase is active and that the subunit interaction of the $\alpha\alpha$ isozyme is not essential for the expression of catalytic activity. The immobilization of the dimer causes a loss of activity of about 25%, and this probably affects subunit interaction.

In 1990, Trepanier et al. (6) used fluorescence polarization of FITC-$\gamma\gamma$ to probe the dissociation by $NaClO_4$. The inactivation and the dissociation were analyzed; they showed that the decrease in activity is a two-step process where the rate of depolarization is faster than inactivation. An active monomer is produced via the dissociation (model [3]).

The fluorescent lifetime of FITC-$\gamma\gamma$ was found to be un-affected during the incubation in $NaClO_4$ which is an important requirement for the fluorescence polarization technique (27, 29).

Recently, in 1993, Kornblatt et al. (30) reported that pressure induces the dissociation of the active dimeric enzyme of the $\gamma\gamma$ enolase into inactive monomers. The following diagram summarizes the process involved (model [2]):



($\gamma\gamma$, the dimeric form of enolase; γ the monomeric form of enolase).

This conclusion was reached knowing that inactivation is a first order process while reactivation obeys second order kinetics.

The contrast between Kornblatt's previous work (5) and the recent work model (30) is due to difficulties in measuring the rate of dissociation of enolase, as the inactivation is irreversible. Moreover, at pressures between 1200 and 2000 bar, no inactivation was detected. Nevertheless, it was possible to see the displacement of the monomer-dimer equilibrium using the technique of subunit scrambling. This result was interpreted as dissociation into active monomers (5).

This work is focused on the rabbit muscle enolase (the $\beta\beta$ isozyme). What is the relationship between the quaternary structure and catalytic activity? Does inactivation always occur via dissociation? Since the preliminary studies by Winstead and Wold (23) on rabbit muscle enolase did not prove their proposed model for the dissociation, more direct evidence about the nature of the subunit dissociation in the

$\beta\beta$ isozyme was required. This may be obtained by monitoring conformational changes, quaternary structure and catalytic activity under conditions that are known to cause dissociation and/or inactivation of enolase (eg. using sodium perchlorate, $NaClO_4$) (6,31,32).

In order to answer the above questions, an analysis of the time and concentration dependency of inactivation and dissociation was performed by incubating the rabbit muscle enolase in NaCl and $NaClO_4$. The intrinsic or extrinsic fluorescence (intensity, polarization, quenching) of proteins is widely used to monitor conformational changes and dissociation of multimeric enzymes. Thus, enolase was first labelled with 5-(dimethylamino) naphthalene-1-sulfonyl chloride (dansyl-Cl) in order to generate the dansyl-$\beta\beta$ species, which was subsequently characterized. This involved dissociation of dansyl-$\beta\beta$ using $NaClO_4$, coupled to a monitoring of changes in the extrinsic fluorescence.

Since rabbit muscle enolase has only 3 tryptophan residues (13), and the fluorescence of $\beta\beta$ enolase is increased on addition of substrate (unpublished data in our laboratory), changes in the intrinsic fluorescence during the inactivation and dissociation of $\beta\beta$ enolase were monitored. Does the change in the intrinsic or extrinsic fluorescence monitor the dissociation or inactivation? This was investigated in the course of the study.

The use of glutaraldehyde as a bifunctional crosslinking reagent has become a powerful tool for analysis of dissociation or reassociation processes (26), and was applied in this study. The $\beta\beta$ isozyme of enolase was incubated with salt, then was

crosslinked with glutaraldehyde and analyzed by SDS-PAGE to detect the dissociation. The relative proportion of dimers and monomers (as determined by densitometry of the stained gel) were measured as a function of the length of incubation in salt and salt concentration. The enzymatic activity was studied under the same conditions.

To answer the question, "does inactivation always occur via dissociation?", the effects of $NaClO_4$ on the activity of $\beta\beta$ enolase were studied in the absence of dissociation. Dissociation can be prevented by covalently crosslinking the enzyme with BS^3 (prior to incubation with salt) or by the addition of substrate (6,20).

It was shown that $NaClO_4$ inactivates and dissociates rabbit muscle enolase. This salt affected the tryptophan fluorescence of the $\beta\beta$ and dramatically increased the fluorescence intensity of dansyl-$\beta\beta$. Inactivation precedes dissociation and it is observed even though dissociation is prevented.

B: MATERIALS AND METHODS

B:0: Basic.

B:0.1: Source of enolase.

Rabbit muscle enolase suspended in ammonium sulfate was purchased from Boehringer Mannheim. The $\beta\beta$ enolase (2 mg/ml) was dialyzed for two hours at 4°C against 200 ml of MTM buffer pH 7.15 containing:

25 mM MES (2[N-morpholino]ethanesulfonic acid),

25 mM TRIS (tris[hydroxymethyl]aminomethane) and

1 mM Magnesium acetate.

The dialysis buffer was then replaced by 200 ml of the same buffer; the enzyme was dialyzed overnight and then stored in 50% glycerol at -20°C.

B: 0.2: Enzyme activity assay.

The assay buffer used in the assay for enolase activity contained:

50 mM Imidazole (pH 7.15)

250 mM KCl

1 mM Magnesium acetate

0.1 mM EDTA

The reaction in assay medium was initiated by addition of 1.0 mM 2-phosphoglyceric acid (PGA). The production of phosphoenolpyruvic acid (PEP) at 25°C was followed spectrophotometrically at 240 nm.

B: 0.3: Protein assay.

The concentration of protein was measured by the Bio-Rad assay, using bovine serum albumin as a standard.

B: 1.1: Labelling of $\beta\beta$ enolase with dansyl-Cl.

Labelling of $\beta\beta$ enolase with dansyl-Cl (5-dimethylaminonaphthalene-1-sulfonyl chloride) was performed according to the procedure of Trepanier (6) and Pin (33) with the following modification: 1ml of $\beta\beta$ enolase (2 mg/ml) was dialyzed for approximately 1 hour at 4°C against 200 ml of HEPES buffer (pH 8.0) containing:

25 mM HEPES
(N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)

100 mM KCl

5 mM Magnesium acetate

0.1 mM EDTA

A second dialysis was performed overnight at 4°C using the same volume of the fresh HEPES buffer. The contents of the dialysis bag were removed and a volume 38μl of 50mM PGA was added. After approximately 5 minutes, 80μl of 4mM dansyl-Cl were added, [10.8 mg of dansyl-Cl (10% in celite purchased from Molecular Probes) suspended in 1 ml of N,N-dimethylformamide] were added. This solution was gently stirred, in the dark, at 25°C for approximately 2 hours. The mixture was then centrifuged for 3 minutes to remove the celite. The supernatant was then placed onto a K 9/30 (9 mm x 30 cm) column of G-25 Sephadex (super fine grade, Pharmacia) equilibrated in MTM buffer and eluted with the same buffer. Fractions

(0.5 ml) were collected and assayed for activity. The active fractions were pooled and dialyzed overnight at 4°C against 1 litre of MTM buffer. One volume of glycerol was subsequently added and the dansyl-$\beta\beta$ solution was stored at -20°C.

B: 1.2: Fluorescence emission spectra of dansyl-$\beta\beta$ and dansyl-Cl.

a/ Dansyl-$\beta\beta$ (90 nM in MTM buffer):

The signal gain was set at 0.3 and the slits were 10 nM wide on a Perkin-Elmer MPF-44 fluorometer with a xenon lamp power supply and the temperature equilibrated at 25°C. The excitation was set at 340nm for the emission spectrum; for the excitation spectrum, the emission was set at 515nm. Slit widths and signal gain varied in other experiments, depending on the concentration of $\beta\beta$ enolase.

b/ Dansyl-Cl:

A solution of dansyl-Cl in MTM buffer plus 0.8M NaCl or 0.8M $NaClO_4$ was prepared by adding dansyl-Cl (which was dissolved in N,N-dimethylformamide) to an MTM buffer plus the salts such that when that the signal gain on the fluorometer was set at 0.3, the intensity equalled that of 90nM dansyl-$\beta\beta$. Excitation and emission were set at 316nm and 500nm respectively.

B: 2.1: Inactivation and dissociation of dansyl-$\beta\beta$ versus time, [NaCl] and [$NaClO_4$].

Inactivation and dissociation of dansyl-$\beta\beta$ were performed in MTM buffer at pH 7.15 with varying concentrations of NaCl or $NaClO_4$. Dansyl-$\beta\beta$ was added to give a final concentration of 90nM in one set of experiments and 300nM in another set. Following incubation at 25°C for varying times (or for 10 and 20 minutes as a function of [NaCl] and [$NaClO_4$]), aliquots were removed and assayed for enzymatic activity or used for a cross-linking experiment.

B: 2.2: Reactivation.

Dansyl-$\beta\beta$ 90nM was first inactivated (by 90% as compared to the control) in MTM buffer containing 1mM DTT (dithiothreitol), 1mM EGTA and 0.4M $NaClO_4$. The inactivated enzyme-salt solution was dialyzed against a similar buffer, minus sodium perchlorate, for 4 hours at room temperature. The contents were removed and assayed for enzymatic activity.

B: 3.1: Tryptophan fluorescence emission of $\beta\beta$ enolase in buffer, NaCl, $NaClO_4$ and guanidine hydrochloride (GdnHCl).

Solutions of $\beta\beta$ enolase (approximately 0.3mM) in MTM buffer plus 0.8M NaCl, 0.8M $NaClO_4$ or 6M GdnHCl were incubated at 25°C for 30 minutes. All samples were then excited at 270nm and the emission was scanned.

B: 3.2: Emission spectra of dansyl-ββ in buffer, NaCl or $NaClO_4$.

Solutions of dansyl-ββ (0.3 μM) in MTM buffer plus 0.8M NaCl or $NaClO_4$ were incubated at 25°C for 60 minutes and emission spectra were recorded with excitation at 340 nm.

B: 3.3: Fluorescence intensity of dansyl-ββ versus time, [NaCl] and [$NaClO_4$].

The procedure was exactly as in (**B:2.1**), except that at varying times the fluorescence intensity of the samples was measured at 515nm (emission) with excitation wavelength at 340nm. The sample compartment's temperature was set at 25°C.

B: 4.1: Crosslinking and SDS-PAGE.

After an incubation performed as described in paragraph (**B: 2.1**), crosslinking by glutaraldehyde was carried out according to the procedure of Burn and Schachman (31), with some modifications. A 150μl aliquot of incubated enolase was placed in a test tube; this was immediately followed by addition of 12μl of 0.25M glutaraldehyde (glutaraldehyde, grade 1, 25% aqueous solution, purchased from Sigma, and diluted 1:10 with water). After one minute of incubation, 20μl of 1M $NaBH_4$ (38mg/ml in 0.1M of NaOH), was added to terminate the reaction. The mixture was left for 20 minutes to eliminate bubbles. Fifty microliters of sample buffer (2x concentration) were then added; the samples were kept at room temperature until completion of the time course.

All of the reaction tubes were then placed in a boiling water bath for 2 minutes. Aliquots of 35μl were removed from each tube and placed into the wells of a standard SDS-PAGE slab gel (12% acrylamide) (34). The protein on the gel was visualized using silver stain (35). The density of the dimer bands in the stained gel was estimated by laser densitometry (LKB, BROMMA, 2202 ULTRO SCAN, laser densitometer).

B: 5.0: BS^3 crosslinking.

Two samples of $\beta\beta$ enolase and dansyl-$\beta\beta$ enolase (2.0mg/ml) (1ml), were incubated for 30 minutes at room temperature with 2mM BS^3 [bis(sulfosuccinimidyl) suberate, purchased from Pierce] (36). The reactions were then quenched by the addition of 7mM ethanolamine. The crosslinked enzyme samples were dialyzed overnight against MTM buffer, diluted with glycerol and stored at -20°C.

C. Results and Discussion

In order to study the relationship between inactivation and dissociation of rabbit muscle enolase, it is necessary to be able to monitor the two processes quantitatively under the same conditions. In addition, conformational changes should be investigated during the inactivation and dissociation of the enzyme.

Here, we focus on the effects of salt on three properties of enolase:

1- Inactivation.

2- Conformational changes.

3- Dissociation.

Inactivation was monitored spectrophotometrically by the activity assay. Intrinsic and/or extrinsic fluorescence changes, if they occur, reflect the conformational changes. Dissociation was monitored using a crosslinking and SDS-PAGE method. After monitoring and comparing the properties under the same conditions, dissociation was prevented and the effects, if any, on inactivation and conformational changes were assessed.

C.1.0: Formation and Characterization of dansyl-$\beta\beta$.

A fluorescent molecule, or probe, facilitates the study of conformational changes in the tertiary and quaternary structures of the enzyme during inactivation and dissociation. For this purpose, the $\beta\beta$ isozyme of rabbit enolase was covalently labelled with dansyl chloride (dansyl-Cl). Dansyl-Cl has been chosen because its

fluorescence has quite high environmental sensitivity (37). Paladini and Weber (14) studied the fluorescence polarization of dansyl chloride conjugates of yeast enolase to measure the dissociation. Moreover dansyl-Cl was a useful probe used to monitor subunit interactions of hemoglobin (33).

C:1.1: Emission and excitation spectra of dansyl-$\beta\beta$ and dansyl-Cl.

The labelling of $\beta\beta$ enolase with dansyl-Cl was carried out according to the procedure outlined in materials and methods. G-25 gel filtration followed by dialysis is assumed to remove any non-covalently bound dansyl-Cl.

The excitation and emission spectra of the dansyl-$\beta\beta$ (Fig. 2) and free dansyl-Cl (Fig. 3) exhibit different excitation and emission maxima. The excitation and emission maxima of dansyl-$\beta\beta$ are 340nm and 515nm respectively. On the other hand, the excitation and emission maxima of dansyl-Cl are 316nm and 500nm respectively. The significant differences in the spectra of the dansyl groups is due to the high sensitivity of the dansyl group to the environment (37,38). The attachment of dansyl to a protein involves a reaction between the sulfonyl chloride group of dansyl-Cl and an unprotonated amine side chain of lysine. Sulfonyl chloride may also react with thiols such as cysteine and imidazoles such as histidine, but the products of these reactions are generally unstable (37).

Figure 2: Excitation and emission spectra of dansyl-$\beta\beta$ in MTM buffer, pH = 7.15 at 25°C.

[dansyl-$\beta\beta$] = 90 nM

*- Excitation spectrum of dansyl-$\beta\beta$.
(emission set at 515 nm)

*- Emission spectrum of dansyl-$\beta\beta$.
(excitation set at 340 nm)

signal gain (S.G.) = 0.3

slits = 10 nM

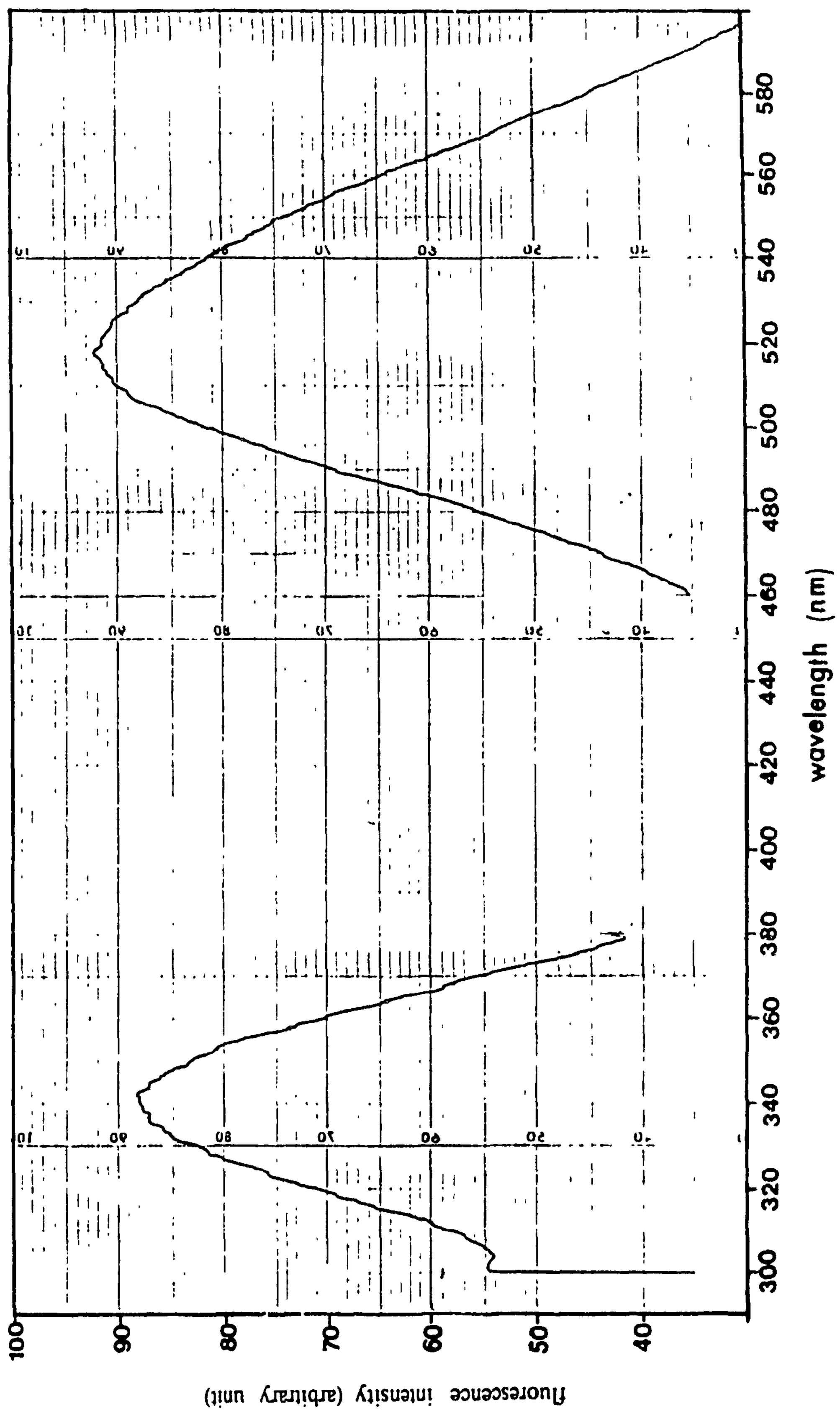

fluorescence intensity (arbitrary unit)
100
90
80
70
60
50
40
wavelength (nm)
300
320
340
360
380
400
420
440
460
480
500
520
540
560
580

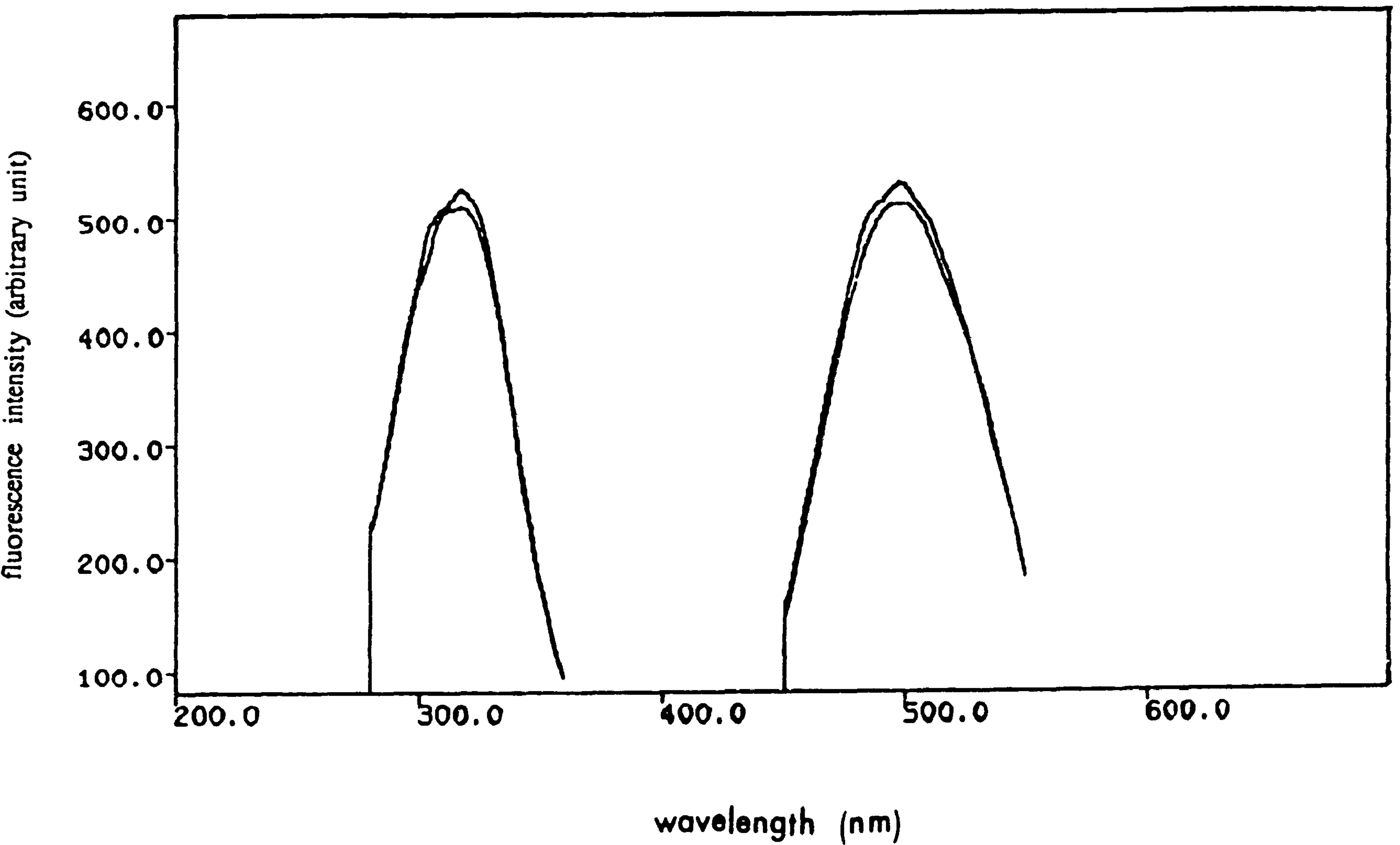
Figure 3: Excitation and emission spectra of dansyl-Cl in MTM buffer plus 0.8M NaCl or 0.8M $NaClO_4$.

*- Emission spectra in 0.8M NaCl and 0.8M $NaClO_4$ (excitation wavelength =316nm).

*- Excitation spectra in 0.8M NaCl and 0.8M $NaClO_4$ (emission wavelength =500nm).

slits = 5 nm

note: the spectra were taken under different condition on different machine.


fluorescence intensity (arbitrary unit)
600.0
500.0
400.0
300.0
200.0
100.0
200.0
300.0
400.0
500.0
600.0
wavelength (nm)

C:1.2: Degree of labelling of $\beta\beta$ enolase and relative specific activity of dansyl-$\beta\beta$.

Based on the absorption of dansyl-$\beta\beta$ at 340nm, and using an extinction coefficient of 4300 $M^{-1}cm^{-1}$ for the dansyl group (39), the degree of labelling was calculated to be 0.85 dansyl groups /dimer. The labelling procedure in several trials yielded a degree of labelling ranging from 0.8 to 1.0 dansyl groups /dimer.

The specific activity of dansyl-$\beta\beta$ is found to be 90-100 % that of native $\beta\beta$ enolase. The dansyl-$\beta\beta$ species was used in all experiments (unless otherwise indicated).

C:2.0: Inactivation of dansyl-$\beta\beta$.

As mentioned previously (see introduction) the interest of this study was to analyze the relationship between inactivation and dissociation of the $\beta\beta$ isozyme and conformational changes occurring under the same conditions. It was important therefore to choose an appropriate reagent which causes the dissociation of the enzyme and which can be used for all experiments.

C:2.1: Choosing the dissociation agent.

Oligomeric enzymes undergo dissociation into subunits, mostly accompanied by inactivation, due to action of various agents (see introduction) (26). The most widely used agents are anions such as SCN^-, ClO_4^-, I^-, Br^-, Cl^-, etc. which dissociate oligomeric proteins at low concentration and denature them at high concentration (26,40). In many cases, denaturation and dissociation have been performed using

compounds such as urea or guanidine hydrochloride, which cause dissociation by a different mechanism. Electrolytes are thought to alter the native conformations of proteins by producing changes in the water structure solvating the protein (*i.e.* act as chaotropic ions by favouring the transfer of apolar group to water) and by preferential interaction with the protein constituents (*i.e.* preferential binding to the dissociated species possibly resulting from the availability of extra binding sites) (40).

Trepanier (1990) used $NaClO_4$ (instead of other salts) to dissociate $\gamma\gamma$ enolase isozyme. He found that $NaClO_4$ at a concentration of 0.5 M dissociates the enzyme. Moreover, it is not a quencher of the lifetime of FITC when it is covalently attached to $\gamma\gamma$ enolase (6,27). In this study, $NaClO_4$ was used as a dissociating agent since it readily dissociates oligomeric enzymes, depending on the concentration applied (6,31,32,40). Furthermore, $NaClO_4$ neither quenches the lifetime of dansyl-Cl nor enhances it. Figure 3 shows the almost identical emission spectra of dansyl-Cl in 0.8M NaCl and 0.8M $NaClO_4$. The next step, then, was to establish the effects of $NaClO_4$ on activity and subunit interactions in dansyl-$\beta\beta$.

C:2.2: Activity of dansyl-$\beta\beta$ in $NaClO_4$ as a function of time and $[NaClO_4]$.

Figure 4 shows the inactivation of dansyl-$\beta\beta$ observed at various time intervals as a function of the $NaClO_4$ concentration. The initial velocities were determined at regular time intervals as a percent of a control incubated in the absence of $NaClO_4$, but in the presence of NaCl. NaCl had no effect on the inactivation of the enzyme (Fig. 4,5). However, Na^+ as well as Li^+ cations act as inhibitory ions by

specific binding to the mammalian and yeast enolases (7,41). Thus all studies of inactivation have been corrected for inhibition by Na^+. Since there was no change in activity upon incubation in NaCl, the average activity (from 0 to 60 min) of the enzyme in NaCl was used as the zero time value for the enzyme in NaCl and in the same concentration of $NaClO_4$. Values for the enzyme in $NaClO_4$ at later times are expressed as % of the zero time value. For inactivation versus [$NaClO_4$], activity of the enzyme in $NaClO_4$ is expressed as % activity relative to enzyme incubated in the same concentration of NaCl.

Enzyme incubated in NaCl and then assayed has the same activity as enzyme incubated in buffer but assayed with a corresponding amount of NaCl added to the assay (unpublished data in our laboratory).

The equilibrium inactivation value was attained within 20 minutes at 0.8M $NaClO_4$. At high concentrations of salt, the equilibrium inactivation was reached at a faster rate than at low concentrations. In other words, the rate of inactivation increases as a result of increase in salt concentration.

The inactivation of dansyl-$\beta\beta$ as a function of $NaClO_4$ concentration is shown in Figure 5. A steady state level of inactivation was reached at approximately 0.8M $NaClO_4$. This indicates that the $\beta\beta$ isozyme is more resistant to inactivation than the $\gamma\gamma$ isozyme , since the inactivation of $\gamma\gamma$ reached equilibrium by 0.5M $NaClO_4$ (6,27).

Figure 4: Activity of dansyl-$\beta\beta$ as function of time in 0 - 1M $NaClO_4$.

[dansyl-$\beta\beta$] = 90nM

● = 0.4M NaCl

▽ = 0.2M $NaClO_4$

▼ = 0.4M $NaClO_4$

□ = 0.6M $NaClO_4$

■ = 0.8M $NaClO_4$

△ = 1.0M $NaClO_4$

Experimental details as per material and methods.

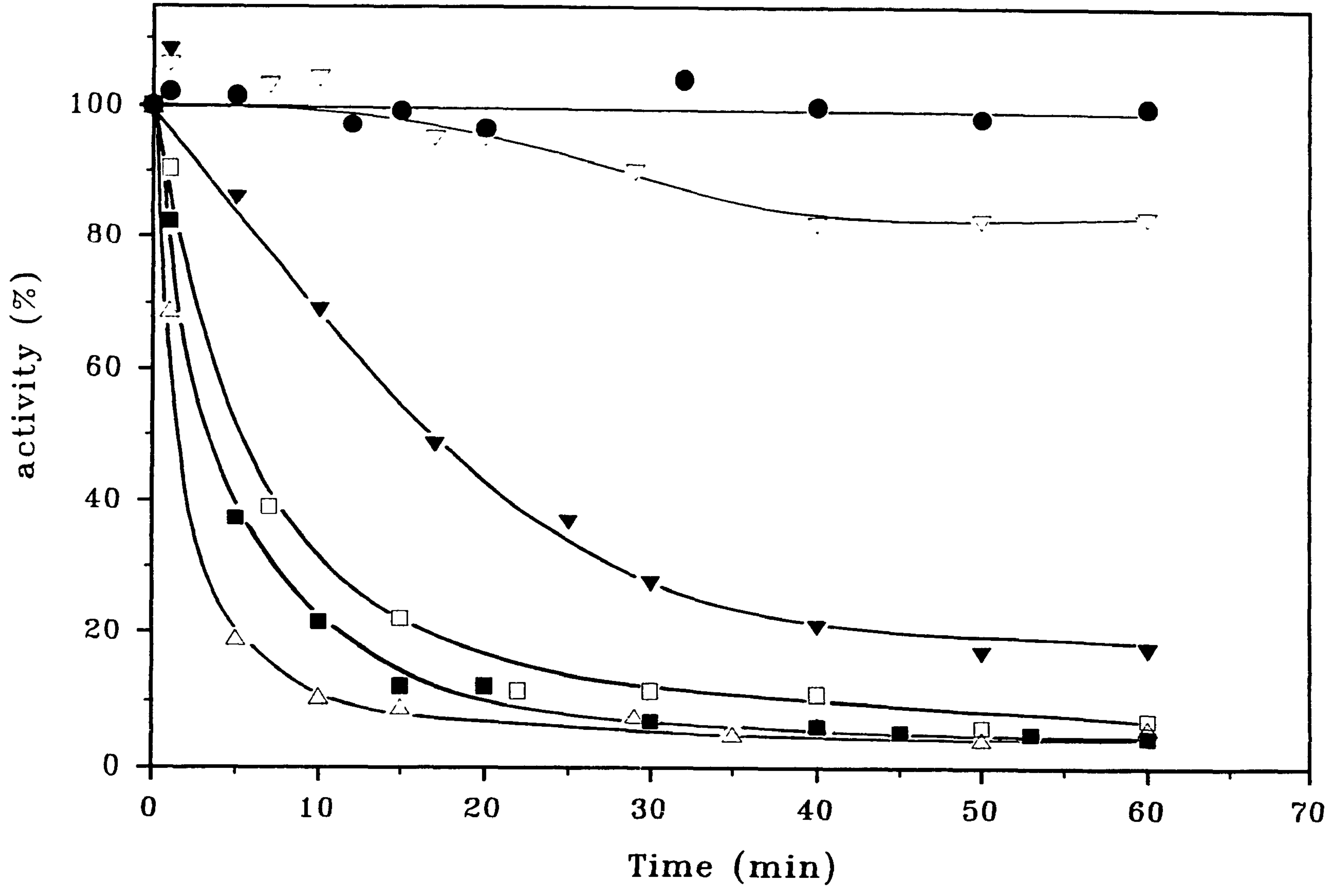

activity (%)
100
80
60
40
20
0
Time (min)
0
10
20
30
40
50
60
70

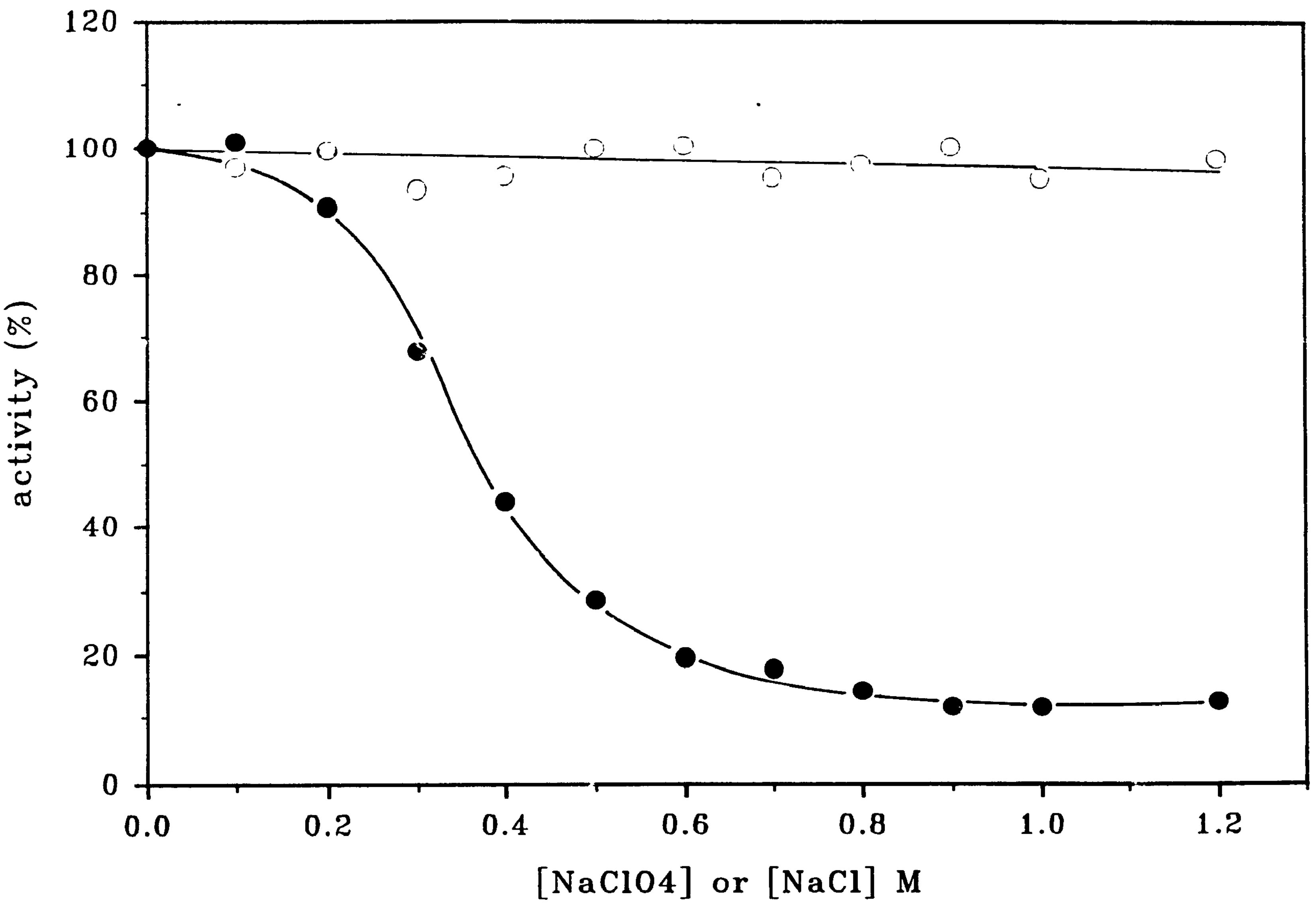
Figure 5: Activity of dansyl-$\beta\beta$ versus [$NaClO_4$] and [NaCl].

[dansyl-$\beta\beta$] = 90nM

○ = NaCl

● = $NaClO_4$

(the incubation time is 20 minutes)


120
100
80
60
40
20
0
activity (%)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
[NaClO4] or [NaCl] M

The results indicate that inactivation by the chaotropic ions is dependent on $NaClO_4$ concentration as well as the incubation time. It is also concluded that the agent causing the inactivation is the perchlorate anion ClO_4^-, since chloride anions have no effect on activity of $\beta\beta$ and $\gamma\gamma$ isozymes (7). At least three independent techniques - polarization, gel filtration, and subunit scrambling - indicated that $\gamma\gamma$ enolase was dissociated by $NaClO_4$ (6). Therefore, it may be concluded that inactivation by chaotropic ions could be due to inactivation of the dimeric enzyme by conformational changes (altered tertiary structure) and/or dissociation into inactive monomers. Conformational change were investigated by fluorescence techniques (29), and dissociation was monitored by crosslinking and SDS-PAGE (26). But first, the reversibility or non-reversibility of the ßß isozyme inactivation was investigated.

C:2.3: Reactivation.

Mammalian enolases differ in their susceptibility to inactivation as well as reactivation (5,6,8,25). Incubation of the $\beta\beta$ isozyme with 0.8M $NaClO_4$ induced a loss of activity greater than 90% (Fig. 4). Salt removal by dilution of the enzyme-salt solution into the salt-free MTM buffer gave 20% of the activity of a control, whereas salt removal by dialysis against salt-free MTM buffer gave 40%. The low recovery upon dilution (20%) compared to dialysis (40%) may be due to fast reassociation, which resulted in aggregation. As mentioned in the Introduction, Marangos (8) studied the inactivation of brain enolases. In his experiments for reactivation, the

addition of 20mM β-mercaptoethanol to inactivation and reactivation buffers increased the percentage of activity recovered. The addition of DTT (dithiothreitol) to the inactivation and reactivation buffers also enhances the reversibility of rabbit muscle enolase inactivation (unpublished data from our lab). The conditions for inactivation and reactivation were optimized by addition of 1mM DTT and 1mM EGTA to MTM buffer and dialysis at room temperature for 4 hours. This procedure increases the recovery of activity to approximately 70% of the control activity (*i.e.* regain of 60%).

The presence of thiol reagents during the inactivation was required for increasing the reactivation but it does not affect the inactivation process. The effects of DTT include preventing the oxidization of sulfhydryl groups in the enzyme during the incubation time; this could account for the increase in recovered activity observed upon its addition. The low recovery of activity (60%) suggest that some of the enzyme was aggregated or in different conformational states, which could not renature to their original conformation. Yeast enolase with 80% of its initial activity was recovered after unfolding in GdnHCl and subsequent refolding, but in the presence of the chaperonin GroE and ATP in the refolding assay result in a complete recovery of the activity (42). Chaperonin GroE facilitates the correct folding of the proteins (43). The activity of the $\gamma\gamma$ isozyme is fully recovered after inactivation by KBr (8), $NaClO_4$ (6), or pressure (30). The different results in recovered activity of enolase isozymes could be due to difference in their structures.

C:3.0: Monitoring of the intrinsic and extrinsic fluorescence changes of the enzyme during the inactivation.

Fluorescence spectroscopy should be a sensitive tool to monitor conformational changes during the inactivation and dissociation of the enzyme. For macromolecules, such as an enzyme , the fluorescent species can be either intrinsic (tryptophan) or extrinsic. Since the lifetime of tryptophan is short and its emission is weak, an extrinsic fluorescent probe (*i.e.* a small fluorescent molecule or probe attached covalently to an enzyme) is generally used for fluorescence studies on macromolecules.

C:3.1: Tryptophan fluorescence emission spectra of $\beta\beta$ in buffer, $NaClO_4$ or GdnHCl.

Figure 6 shows the fluorescence emission spectra of the $\beta\beta$ isozyme in MTM buffer plus 0.8M NaCl, 0.8M $NaClO_4$ or 6M GdnHCl. The peak at 300 nm is a Raman peak due to the solvent (see fig. 6, x, emission spectrum for the buffer only) (29). The emission maximum for tryptophan in water is 350 nm (29). Thus, the emission maximum for tryptophan was shifted under denaturing conditions from wavelength 328nm to 355nm. Moreover, a decrease in fluorescence intensity was observed for tryptophan. The higher energy emission maximum for $\beta\beta$ enolase in buffer results from an incomplete exposure of all three tryptophans (14) in a less polar environment (as opposed to a complete exposure to the buffer). The average

Figure 6 : Tryptophan fluorescence emission spectra of $\beta\beta$ in buffer plus NaCl, $NaClO_4$ or GdnHCl.

$[\beta\beta]$ = 0.3 mM

Incubation time = 30 minutes

● = Emission in MTM buffer + 0.8M NaCl

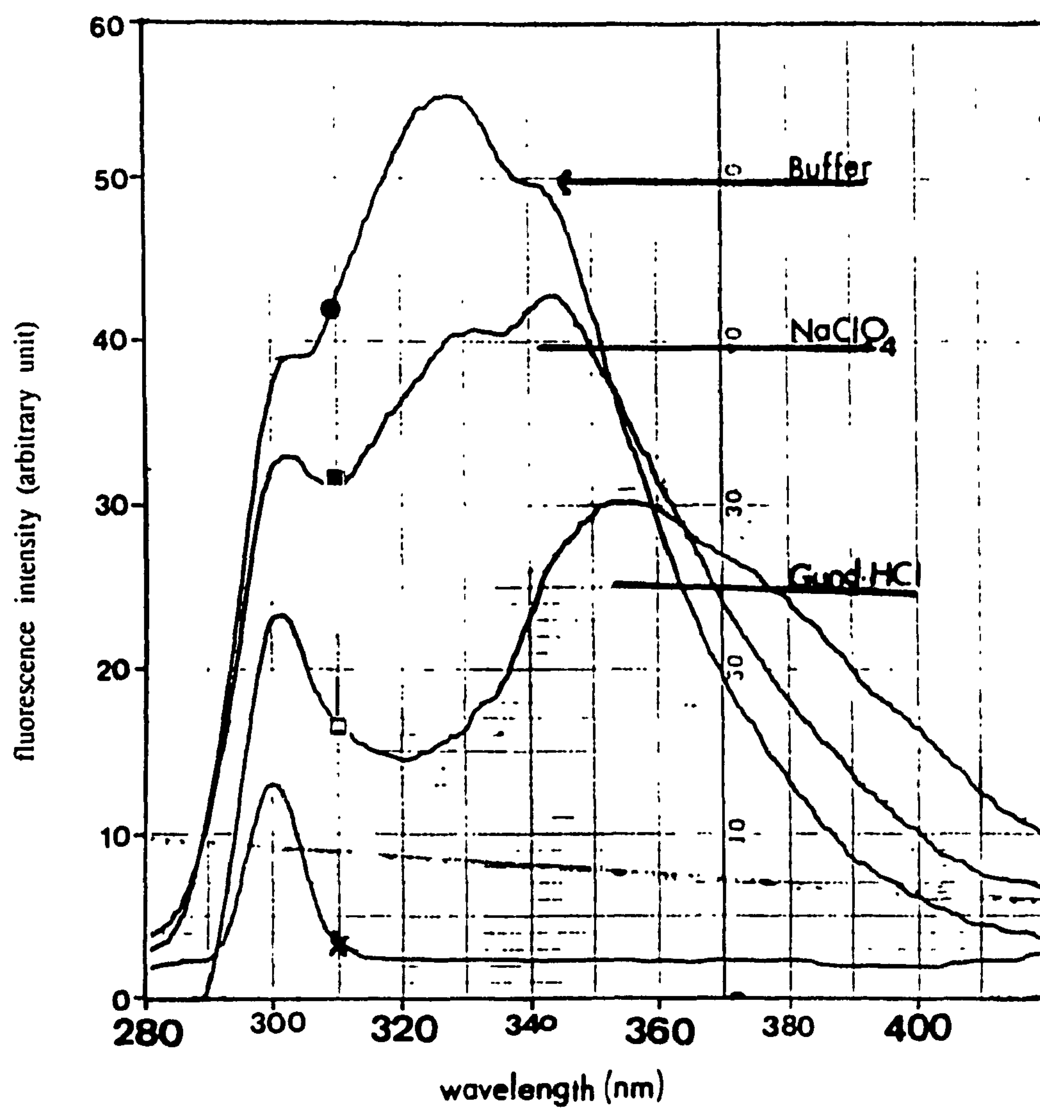
■ = Emission in MTM buffer + 0.8M $NaClO_4$

□ = Emission in MTM buffer + 6M GdnHCl

x = Emission of MTM buffer only.

Excitation =270 nm

Slits = 5 nm



60
50
40
30
20
10
0
280
300
320
340
360
380
400
fluorescence intensity (arbitrary unit)
wavelength (nm)
Buffer
NaClO4
Guad·HCl

tryptophan environment for $\beta\beta$ enolase in 6M GdnHCl is more polar (exposed to solvent) than the native state.

The changes of emission maximum, a slight red shift (3-4nm) and a decreased intensity of $\beta\beta$ enolase were observed after incubation in 0.8M $NaClO_4$ (Fig. 6). The emission spectrum of tryptophan alone is unchanged by the presence of 0.5M $NaClO_4$ (27). Therefore the changes in intensity of the tryptophan emission spectrum of $\beta\beta$ enolase in the presence of $NaClO_4$ must be due to alterations in the environment of one or more tryptophan residues (44).

Paladini and Weber (14) concluded that the red shift and a decrease in the tryptophan fluorescence emission spectra of yeast enolase at 1M KCl or at a pressure of 2 Kilobar, are intrinsic to dissociation. Likewise, Trepanier (27) concluded that dissociation causes the red shift and the decrease in intensity of the $\gamma\gamma$ isozyme's tryptophan emission. However, the red shift and decrease in intensity of the emission spectra of $\beta\beta$ could be due to conformational changes in the tertiary structure of the enzyme as well as dissociation. This would explain the loss of activity and the low percent of activity recovered.

C:3.2: Fluorescence emission spectra of dansyl-$\beta\beta$ in MTM buffer plus NaCl or $NaClO_4$.

Dansyl was chosen as a fluorescence probe covalently attached to the $\beta\beta$ isozyme (see C:1.0). The emission spectrum of dansyl-$\beta\beta$ in the presence of $NaClO_4$ (Fig. 7) shows a dramatic increase of fluorescence intensity and a blue shift from

Figure 7 : fluorescence emission spectra of dansyl-$\beta\beta$ .n the absence or presence of $NaClO_4$.

[dansyl-$\beta\beta$] = 0.3 mM

Incubation time = 60 minutes

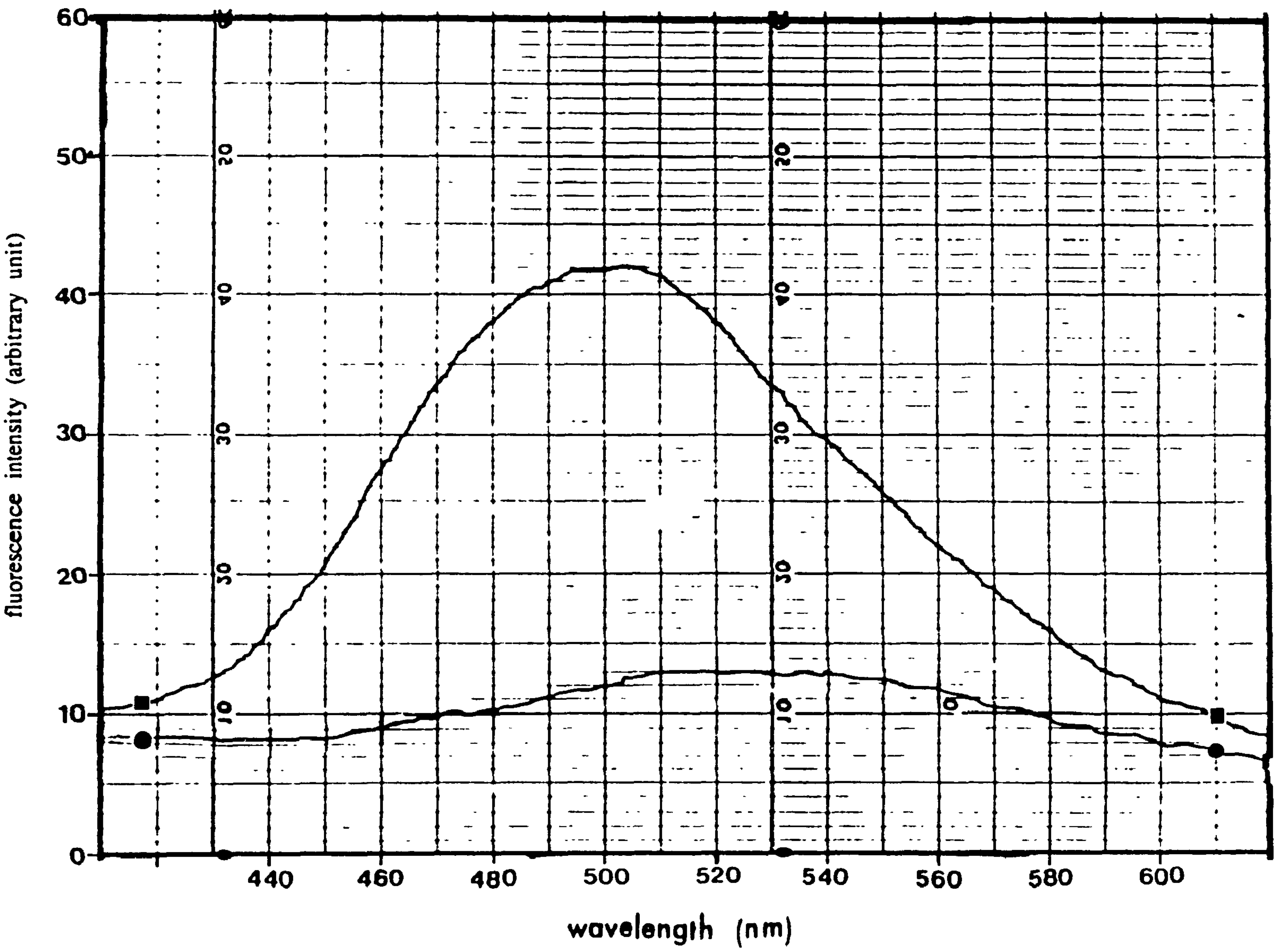
● = emission spectrum in MTM buffer + 0.8M NaCl

■ = emission spectrum in MTM buffer + 0.8M $NaClO_4$

Excitation = 340 nm

Signal gain (S.G) = 0.3

slits = 10 Ex. , 5 Em.


fluorescence intensity (arbitrary unit)
wavelength (nm)
0
10
20
30
40
50
60
440
460
480
500
520
540
560
580
600

515nm in buffer plus 0.8M NaCl to 500 nm in buffer plus 0.8M $NaClO_4$. The increase of fluorescence intensity of dansyl-$\beta\beta$ is due to the anion ClO_4^- since neither Na^+ nor Cl^- affects the fluorescence emission spectra of $\beta\beta$ enolase. The effects of $NaClO_4$ are specific to the protein, not to the fluorophore dansyl (Fig. 3), and therefore it must result from change in the protein environment surrounding the fluorophore. The blue shift to a shorter wavelength (500 nm) in $NaClO_4$ could be due to the average dansyl environment which is now less polar than the native state. The increased intensity is expected to be due to an increase in the lifetime of dansyl caused by a decrease in quenching. The question then is whether the blue shift and the increase of fluorescence of dansyl is due to possible aggregation of protein, or adsorption of the protein to the surface of the cuvette, or whether it is intrinsic to the processes of dissociation and conformational changes surrounding the fluorophore.

The gel filtration elution volume of dansyl-$\beta\beta$ in buffer is larger when incubated and eluted with 0.6M $NaClO_4$ than when it is not. This suggests that there is no aggregation of the protein. Similarly, we have observed increases in intensity with a low concentration of enolase (90 nM) (Fig. 8). The increase of dansyl intensity could not be due to adsorption by the cuvette's surface because the work was done using a low concentration of protein and there was no difference in the intensity if BSA (bovine serum albumin) was added prior to addition of the enzyme.

Thus, it can be concluded that the increase in the intensity of emission of dansyl-$\beta\beta$ in $NaClO_4$ is intrinsic to the processes of dissociation and/or conformational changes induced by $NaClO_4$, which cause the average environment

surrounding the dansyl to become less polar and less quenching. The fluorescence intensity of dansyl conjugated to an amino acid increases as result of a decrease in the dielectric constant of the solvent (38).

The next step was to determine whether the change of dansyl-$\beta\beta$ intensity was time and [$NaClO_4$] dependent. In other words, does the fluorescence of dansyl-$\beta\beta$ behave in the same way as the inactivating process? The answer to this question should provide more conclusive data which would confirm that the effects of $NaClO_4$ on the emission of dansyl-$\beta\beta$ are not directly dependent on the fluorophore, but on the protein environment.

C:3.3: Fluorescence intensity of dansyl-$\beta\beta$ versus time,[NaCl] and [$NaClO_4$].

The fluorescence intensity of dansyl-$\beta\beta$ was determined at various time intervals as a function of the $NaClO_4$ concentration (Fig. 8). The fluorescence intensity increased as the concentration of $NaClO_4$ or the length of incubation time was raised. The achievement of an equilibrium fluorescence value was observed within 20-30 minutes at 0.8M $NaClO_4$. The highest percent increase of intensity was seen within 10-15 minutes. So it was concluded that $NaClO_4$ effects on the fluorescence of dansyl-$\beta\beta$ reach a plateau at 0.8M $NaClO_4$. These results indicate that the percent florescence intensity increases are dependent on $NaClO_4$ concentration as well as incubation time (Fig. 8). NaCl had no effect on the fluorescence of dansyl-$\beta\beta$ (Fig. 8, ●) even at high concentration of salt (0.8M).

Figure 8: Fluorescence intensity of dansyl-$\beta\beta$ as function of time and [$NaClO_4$].

[dansyl-$\beta\beta$] = 90 nM

● = 0.8 M NaCl

▽ = 0.2 M $NaClO_4$

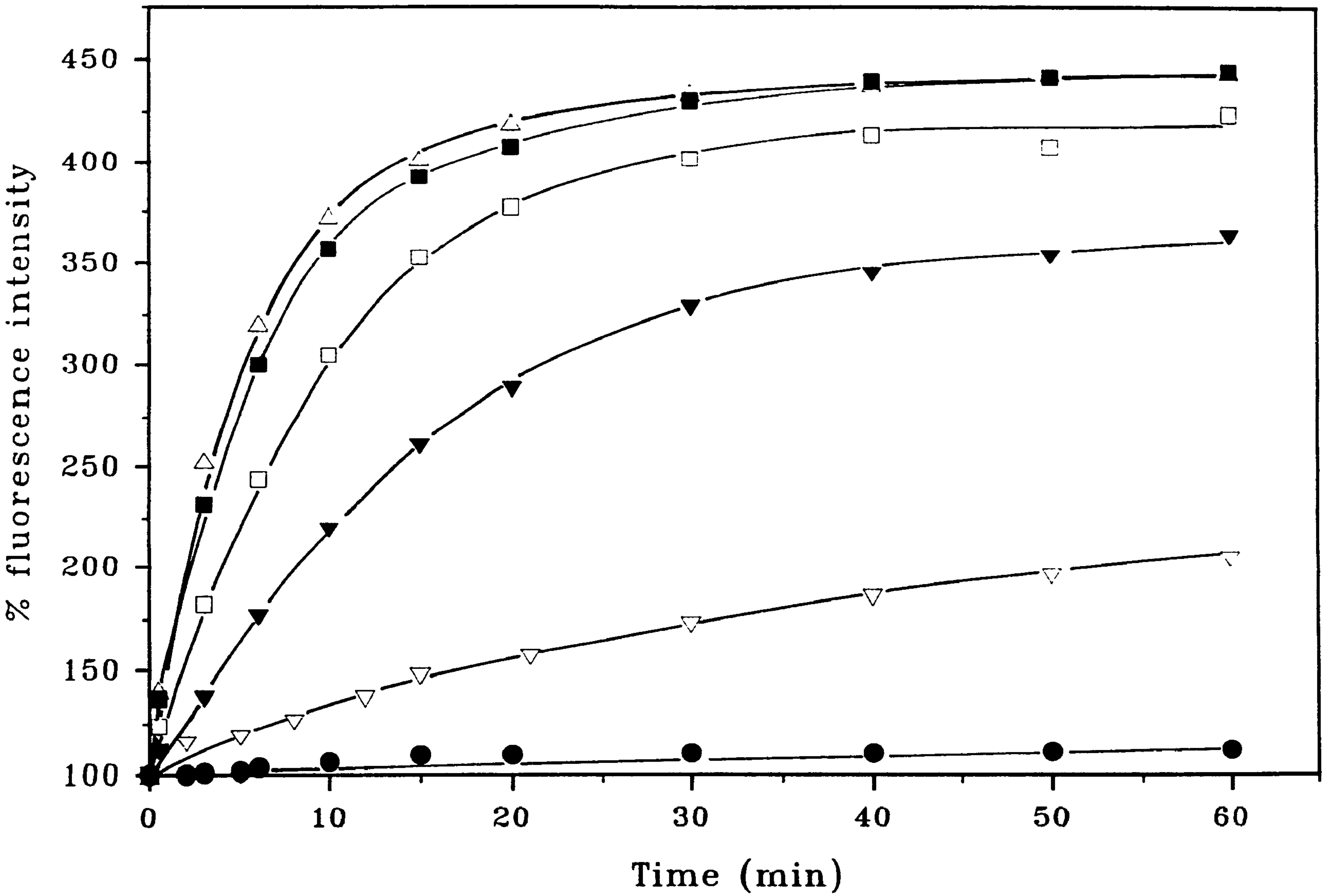
▼ = 0.4 M $NaClO_4$

□ = 0.6 M $NaClO_4$

■ = 0.8 M $NaClO_4$

△ = 0.9 M $NaClO_4$

The control is the same as in inactivation experiments and more experimental details as per materials and methods.



450
400
350
300
250
200
150
100
% fluorescence intensity
0
10
20
30
40
50
60
Time (min)

There are similarities between these results and the effects of $NaClO_4$ and NaCl on the activity of $\beta\beta$ isozyme (Fig. 4). The increase in fluorescence intensity may be due exclusively to conformational changes in the enzyme which then cause the inactivation, or it may be due to dissociation which would alter the tertiary structure and cause an increase in the intensity of the labelled probe, dansyl. To distinguish between these possibilities, it is necessary to quantitatively monitor dissociation and compare the rates of dissociation and inactivation as well as the rate of increase in fluorescence intensity.

C:4.0: Dissociation of dansyl-$\beta\beta$

Sodium perchlorate, $NaClO_4$, was found to be an effective salt for dissociation of multimeric proteins such as sesame α-globulin (32) and hemocyanin (45). Furthermore, there are reports that $\gamma\gamma$ enolase is dissociated by $NaClO_4$; this was confirmed by at least three independent techniques: polarization, gel filtration, and subunit scrambling (6). $NaClO_4$ was chosen as a dissociation agent for the $\beta\beta$ isozyme (see C:2.1). A sensitive and rapid method was needed to quantitatively monitor the dissociation rate. Therefore, crosslinking and SDS-PAGE (Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis) methods were used. These techniques have become powerful tools for the analysis of assembly and dissociation processes (5,26,31).

C:4.1: Dissociation of $\beta\beta$ as monitored by crosslinking and SDS-PAGE.

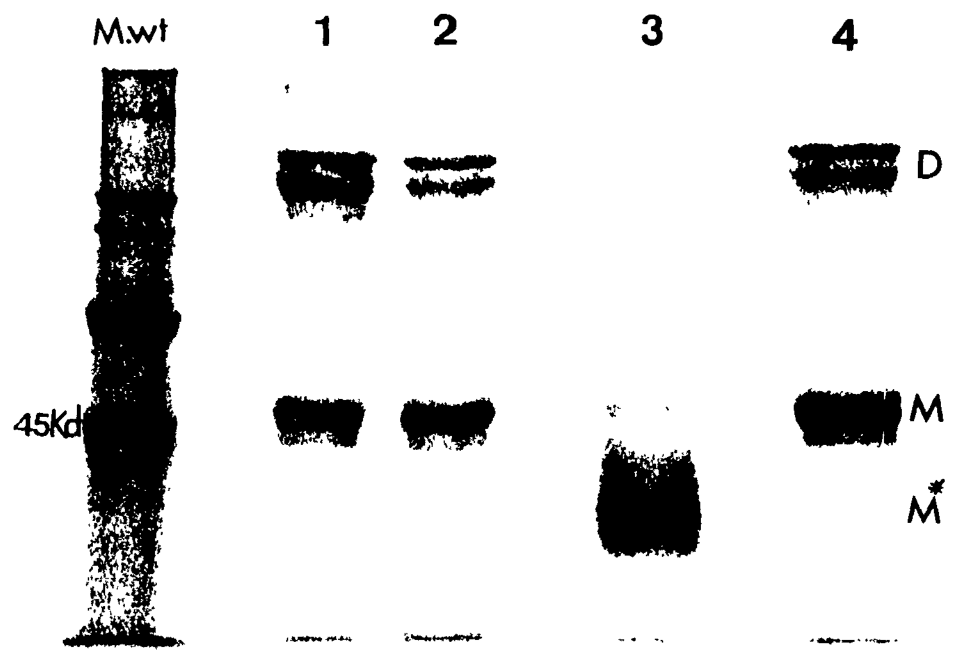
When added to enolase incubated with $NaClO_4$, the bifunctional crosslinking agent glutaraldehyde will covalently link any dimer present through lysine residues on adjacent monomer subunits. The chance of linking two dissociated monomers is assumed to be negligible. The reaction mixture is then analyzed using SDS-PAGE. The SDS denatures and imparts a large negative charge to the protein which then migrates towards the anode of the apparatus according to its molecular weight. Due to the internal crosslinking of the subunits by glutaraldehyde, denaturation by SDS will not dissociate the crosslinked dimer and consequently the denatured dimers will migrate through the gel separately from the uncrosslinked and denatured monomers. Subsequently, the proteins present are visualized using a Coomassie Brilliant Blue protein stain or silver stain.

Enolase was incubated in 0.6M NaCl for 30 minutes; an aliquot was removed and immediately treated with glutaraldehyde (Fig. 9, lane 1) resulting in the formation of a partially crosslinked dimer (D). As the efficiency of crosslinking is not 100%, upon SDS treatment, which causes denaturation and dissociation, the uncrosslinked dimers migrate as monomers (M). Addition of enolase to a solution of 0.6 M $NaClO_4$ followed immediately by treatment of an aliquot with glutaraldehyde, gave similar results (Fig. 9, compare lane 1 to lane 2). After incubation in $NaClO_4$ for 30 minutes prior to addition of glutaraldehyde, almost no crosslinked dimers were formed (lane 3), indicating that dissociation had occurred.

Figure 9: Crosslinking and SDS polyacrylamide gel electrophoresis of the $\beta\beta$ isozyme for the monitoring of dissociation.

	$[\beta\beta]$	=	$0.8\mu M$
lane	Mwt.	=	Molecular weight range 200 Kd - 45 Kd
lane	1	=	30 minutes in 0.6M NaCl followed by crosslinkage.
lane	2	=	0 minutes in 0.6M $NaClO_4$ followed by crosslinkage.
lane	3	=	30 minutes in 0.6M $NaClO_4$ followed by crosslinkage.
lane	4	=	30 minutes in 0.6M $NaClO_4$ + 1mM PGA followed by crosslinkage.

D	=	dimer
M	=	native monomer
M^*	=	internally crosslinked monomer


M.wt
1
2
3
4
D
M
M*
45Kd

In lane 3, the protein migrated as a diffuse band (M*) which migrates more rapidly than the native monomer. Figure 10 shows the effect of internal crosslinkage on the mobility of the monomers. The band in lane 1 (Fig. 10) is the same as the one in lane 3 (Fig. 9), with both obtained by addition of glutaraldehyde after the dissociation. On the other hand, the band in lane 2 (Fig. 10) represents the dissociated monomers obtained by direct SDS-PAGE analysis without glutaraldehyde addition.

The presence of the substrate (PGA) during the $NaClO_4$ incubation (Fig. 9, lane 4) prevents dissociation. Gawronski et al. (20) found that the presence of substrate favours the dimer form of the enzyme by the preventing the inactivation of yeast enolase by potassium chloride. The effect of substrate on dissociation will be considered in more detail later.

Three bands can be recognized in the gel (Fig. 9). The top one is the slower moving band which represents the crosslinked dimer (D); the second one is the native monomer band (M) and the third band is the diffuse band (M*) which probably represents internally crosslinked dissociated monomer. It is easy to distinguish between the dissociation due to SDS denaturation and the dissociation which occurs as a result of incubation with $NaClO_4$ done before to addition of glutaraldehyde and SDS, since the latter induces the diffuse band (Fig. 9, lane 3).

The results in Figure 9 show several features which allow the use of this method to determine the dissociation rate in the presence of $NaClO_4$. As can be seen in lane 1, NaCl has no effect on dissociation, so lane 1 may be used as a control

Figure 10: Dissociation of $\beta\beta$ enolase in 0.6M $NaClO_4$ followed by SDS-PAGE or crosslinking and SDS-PAGE.

[$\beta\beta$] = 0.3μM

lane 1 = 30 minutes in $NaClO_4$ followed by crosslinkage with glutaraldehyde

lane 2 = 30 minutes in $NaClO_4$, no glutaraldehyde

(stained with Coomassie Blue)

1

2

in the dissociation experiment. The short time interval (less than 1 minute) between the enolase addition to the $NaClO_4$ and the subsequent removal of an aliquot to which glutaraldehyde is added, is negligible in comparison to the slow rate of dissociation (lane 2). The relative proportions of the decrease in intensity of the dimer bands (as determined by densitometry of the stained gel) can be measured as a function of the length of incubation with salt or of salt concentration.

We can conclude from the results in Fig. 9 that crosslinking and SDS-PAGE is a useful technique for detecting the dissociation of $\beta\beta$ enolase. In the remainder of the SDS-PAGE experiments, the protein was visualized using silver stain. The relative density of silver stain is linear with protein concentration for some proteins (46).

Is density of the stained bands proportional to the amount of protein (enolase)? This question was answered by preparing various solutions of dansyl-$\beta\beta$ with concentrations ranging from 50 to 300 nM in MTM buffer; aliquots from each preparation were then analyzed by SDS-PAGE and visualization by silver staining. The linear relationship between density of the silver stain and enolase concentration is illustrated the data presented in Figure 11.

It is to be noted that during the dissociation, the concentration of dimer will decrease while the concentration of glutaraldehyde will be constant and in excess. Thus, it should be assumed that the proportion of dimer that crosslinks is independent of the concentration of dimer. To confirm this assumption, varying concentrations of dansyl-$\beta\beta$ ranging from 25 to 400 nM were incubated in the

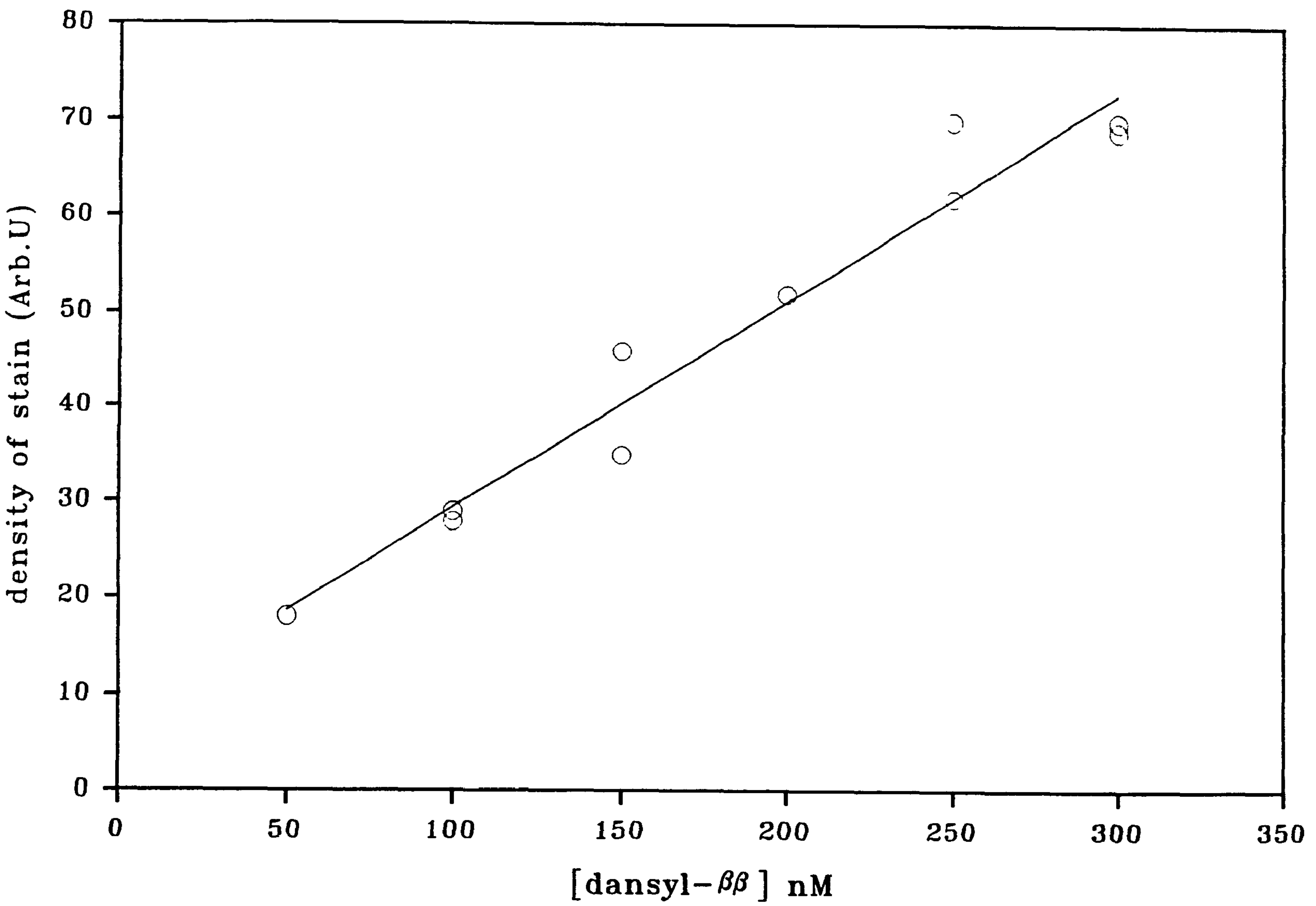
Figure 11: Proportion of the density of the stained bands to the amount of protein.

[dansyl-$\beta\beta$] = 50 nM - 300 nM

Incubation buffer = MTM buffer pH 7.15

- analysis by SDS-PAGE and the determination of the density of bands was described in materials and methods.

(Note: crosslinking with glutaraldehyde was not performed for this experiment)



density of stain (Arb.U)
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
350
[dansyl−ββ] nM

MTM buffer and subjected to crosslinking and SDS-PAGE. The results of this experiment (Fig. 12) show that the density of the stained bands of crosslinked dimer is proportional to the concentration of enzyme (dimer). So, we can conclude that the assumption is correct.

C:4.2: Dissociation of dansyl-$\beta\beta$ in $NaClO_4$, as a function of time and $[NaClO_4]$.

The addition of glutaraldehyde at varying times during enolase incubation with $NaClO_4$ allows for the monitoring of the dissociation as a function of time. Figure 13 shows the time course of dansyl-$\beta\beta$ dissociation in 0.4M $NaClO_4$; glutaraldehyde is added to an aliquot of this mixture at various time intervals. Lanes 1 and 2 represent the enzyme in 0.4M $NaClO_4$ in the presence of 1mM PGA; glutaraldehyde was immediately added to prevent dissociation by both the substrate and glutaraldehyde (see Fig. 9 lanes 2 and 4). The bands in lanes 1 and 2 were used as controls (100%) for the experiment. Lanes 3 to 10 represent a time course for incubation of $\beta\beta$ enolase in the presence of $NaClO_4$. The density of the dimer bands decreases as the length of incubation time in $NaClO_4$ increases. The intensity of dimer band was analyzed by laser densitometry, and the data plotted in Figure 14. The data presented in Figure 14 indicate that the degree of dissociation increases with time.

Figure 12: Proportion of density of the stained bands of crosslinked dimer to the concentration of dimer.

[dansyl-$\beta\beta$] = 25nM - 400nM

Incubation buffer = MTM buffer pH 7.15

- analysis by SDS-PAGE and the determination of the density of bands was described in materials and methods.

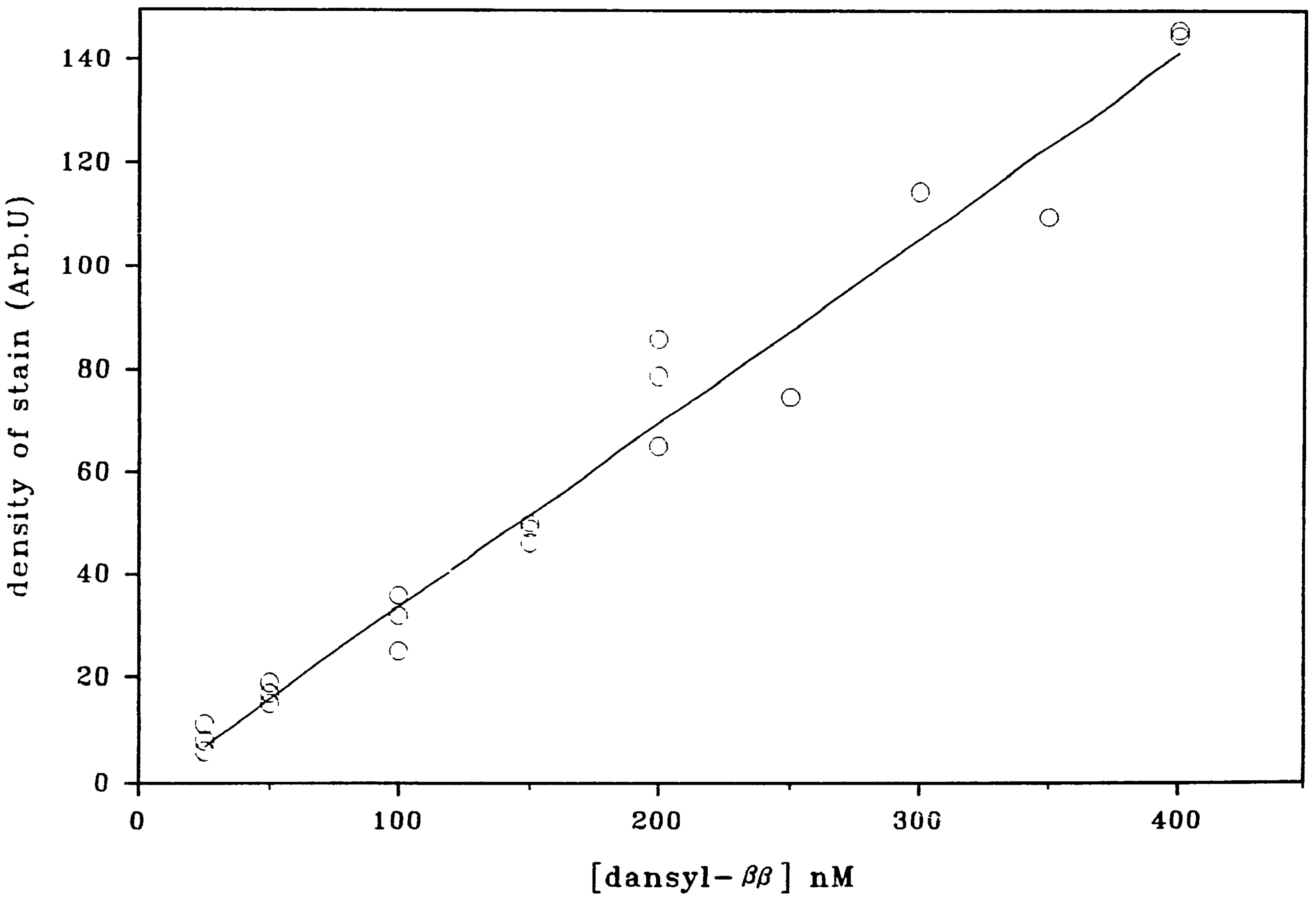
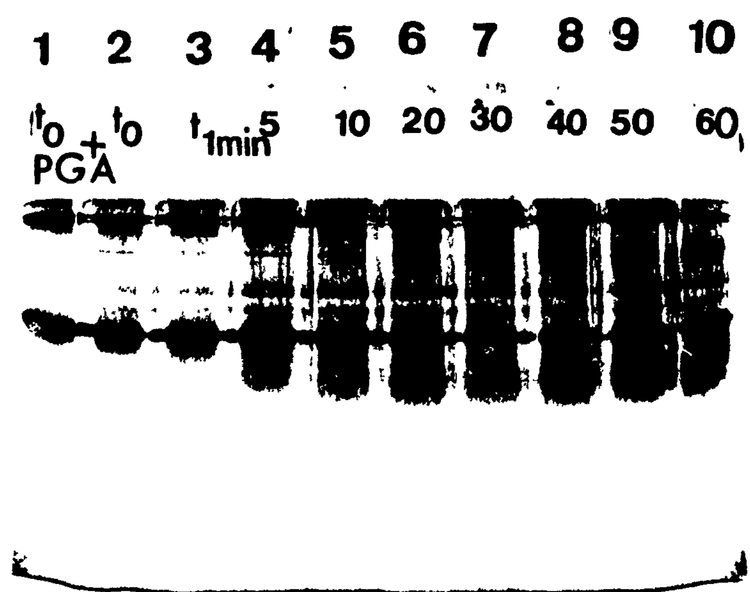

density of stain (Arb.U)
140
120
100
80
60
40
20
0
0
100
200
300
400
[dansyl-$\beta\beta$] nM

Figure 13: Time course of the dissociation of dansyl-$\beta\beta$ in 0.4M $NaClO_4$ as monitored by crosslinking and SDS-PAGE.

[dansyl-$\beta\beta$] = 0.3μM

lanes 1 and 2 = 0 minutes + PGA

lanes 3-10 = 1 minute - 60 minutes as indicated


1 2 3 4 5 6 7 8 9 10
t0 + t0 t1min 5 10 20 30 40 50 60
PGA

Dansyl-$\beta\beta$ was incubated with various concentrations of $NaClO_4$ for 10 minutes, then aliquots were removed and used for crosslinking and SDS-PAGE. The results indicate that dimer bands decreased as the $NaClO_4$ concentration increased (Fig. 15). It can be concluded that dissociation is dependent on $[NaClO_4]$ as well as length of incubation time. Due to variations in the concentration of $NaClO_4$ in the samples, it is possible that higher concentrations of salt affect the crosslinking reaction itself. To test this possibility, enolase was incubated and crosslinked as above but 1mM PGA was added prior to the enzyme additions. Figure 16 shows that the proportion of dimeric enzyme that crosslinks is independent of the concentration of $NaClO_4$ when dissociation is prevented.

Since there is little change in the quantity of crosslinked dimer in the presence of 0.4M NaCl (Fig. 14, ▼) and there is no decrease in the percentage of the dimer in the presence of 0.2-1.4M NaCl (Fig. 15 ▼) it can be concluded that the causative dissociating agent is the perchlorate anion (which also inactivates $\beta\beta$ enolase as shown in Fig. 4 and 5) and that dissociation is independent of ionic strength. Similar results were obtained by Trepanier et al. who showed that NaCl, in contrast to perchlorate anions, does not cause dissociation and inactivation of the $\gamma\gamma$ isozyme (5, 27). Dissociation of the dimer is the result of the increased lipophilicity of water (40). In the presence of chaotropic reagents, the organized hydrogen bonding pattern of water is disrupted, and that increases lipophilicity of water. This increase in lipophilicity weakens the hydrophobic bonds of the enzyme. These hydrophobic

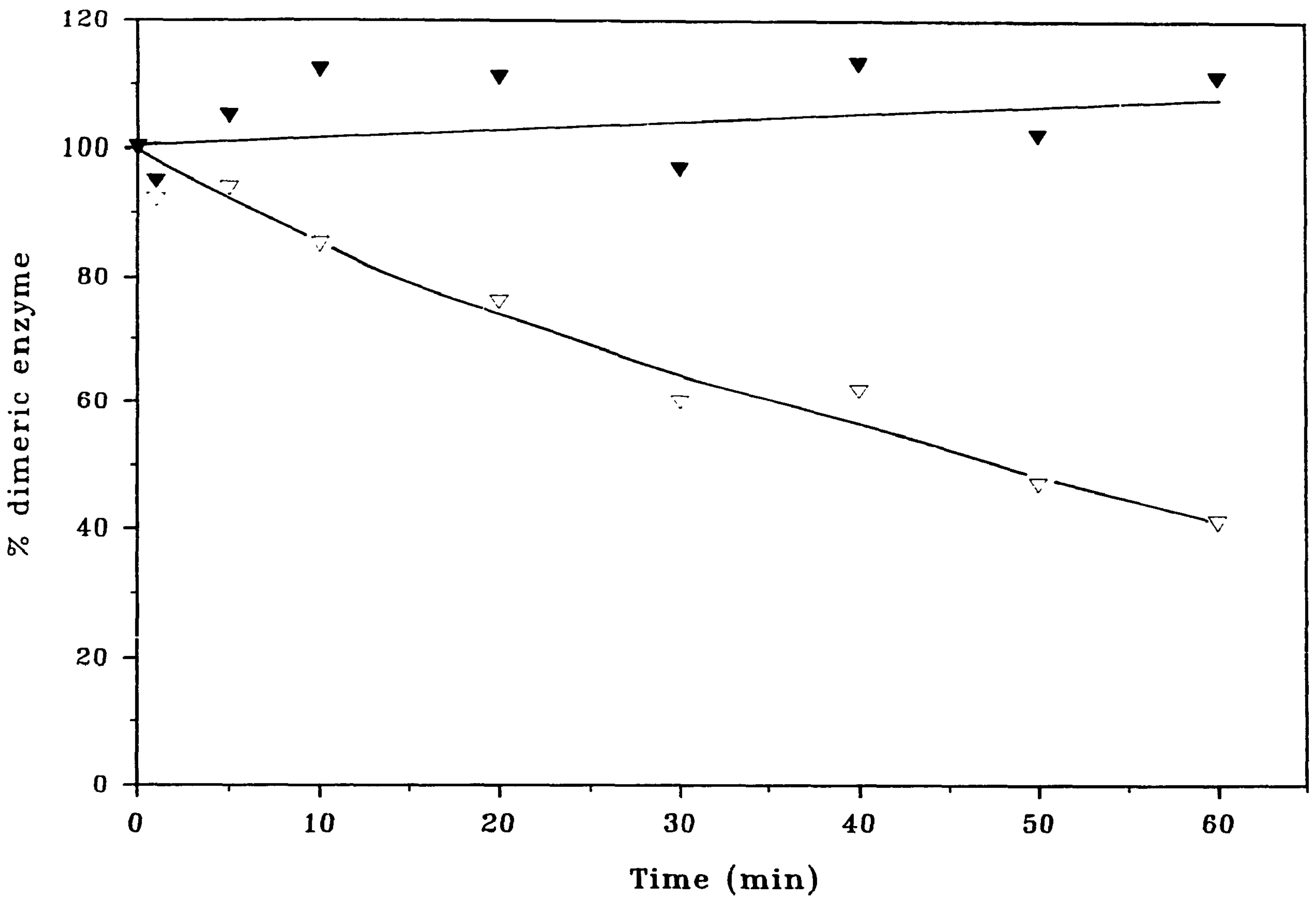
Figure 14: Dissociation of dansyl-$\beta\beta$ in 0.4M $NaClO_4$ as function of time.

[dansyl-$\beta\beta$] = 0.3μM

▼ = % dimeric enzyme in 0.4 NaCl

▽ = % dimeric enzyme in 0.4 $NaClO_4$

Experimental details as per materials and methods.



% dimeric enzyme
0
20
40
60
80
100
120
Time (min)
0
10
20
30
40
50
60

Figure 15: Dissociation of dansyl-$\beta\beta$ versus $NaClO_4$ and NaCl.

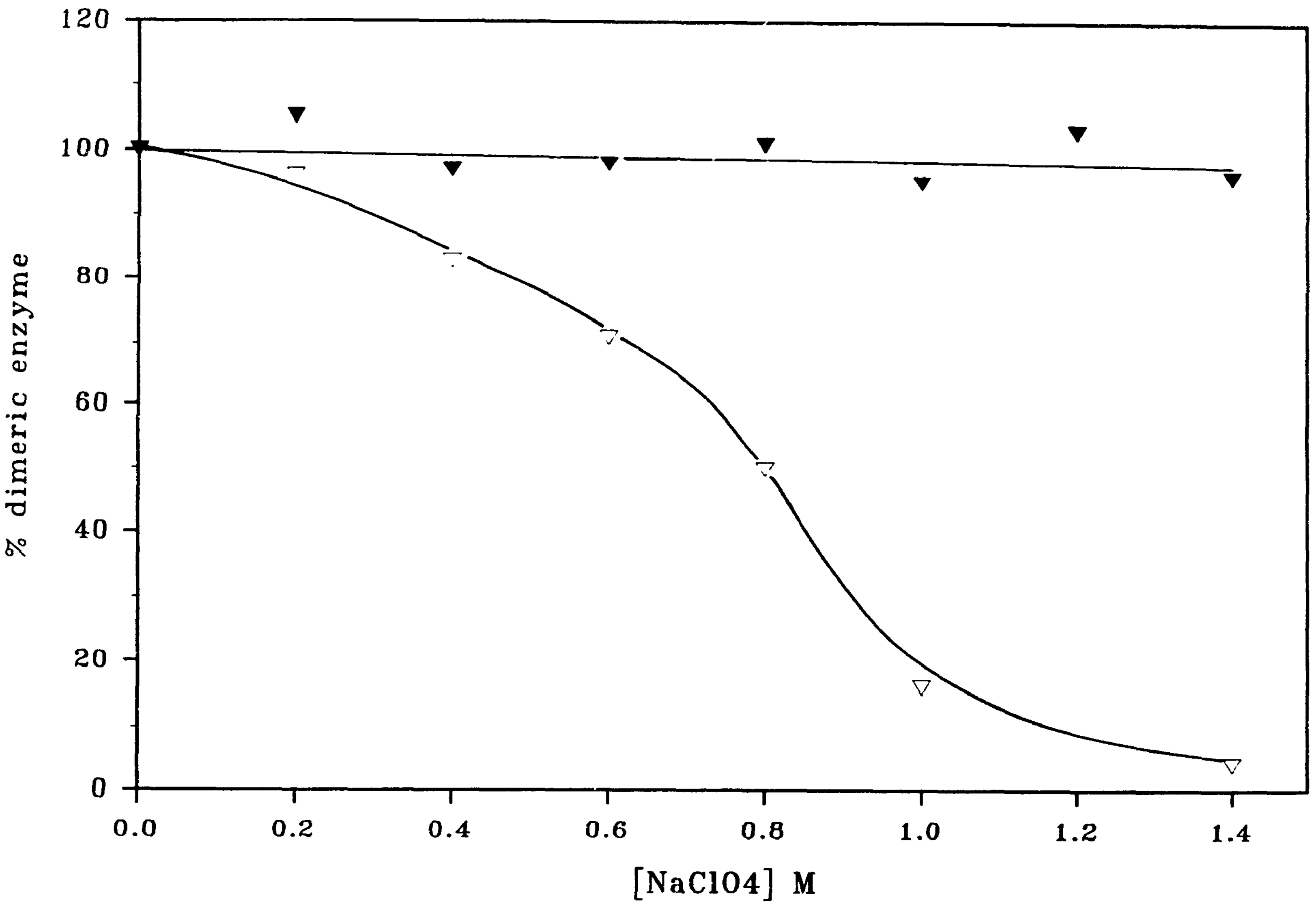
[dansyl-$\beta\beta$] = $0.3\mu M$

Incubation time = 10 min.

▼ = % dimeric enzyme in NaCl

▽ = % dimeric enzyme in $NaClO_4$

Note: 100% of dimeric enzyme is dansyl-$\beta\beta$ in MTM buffer only.



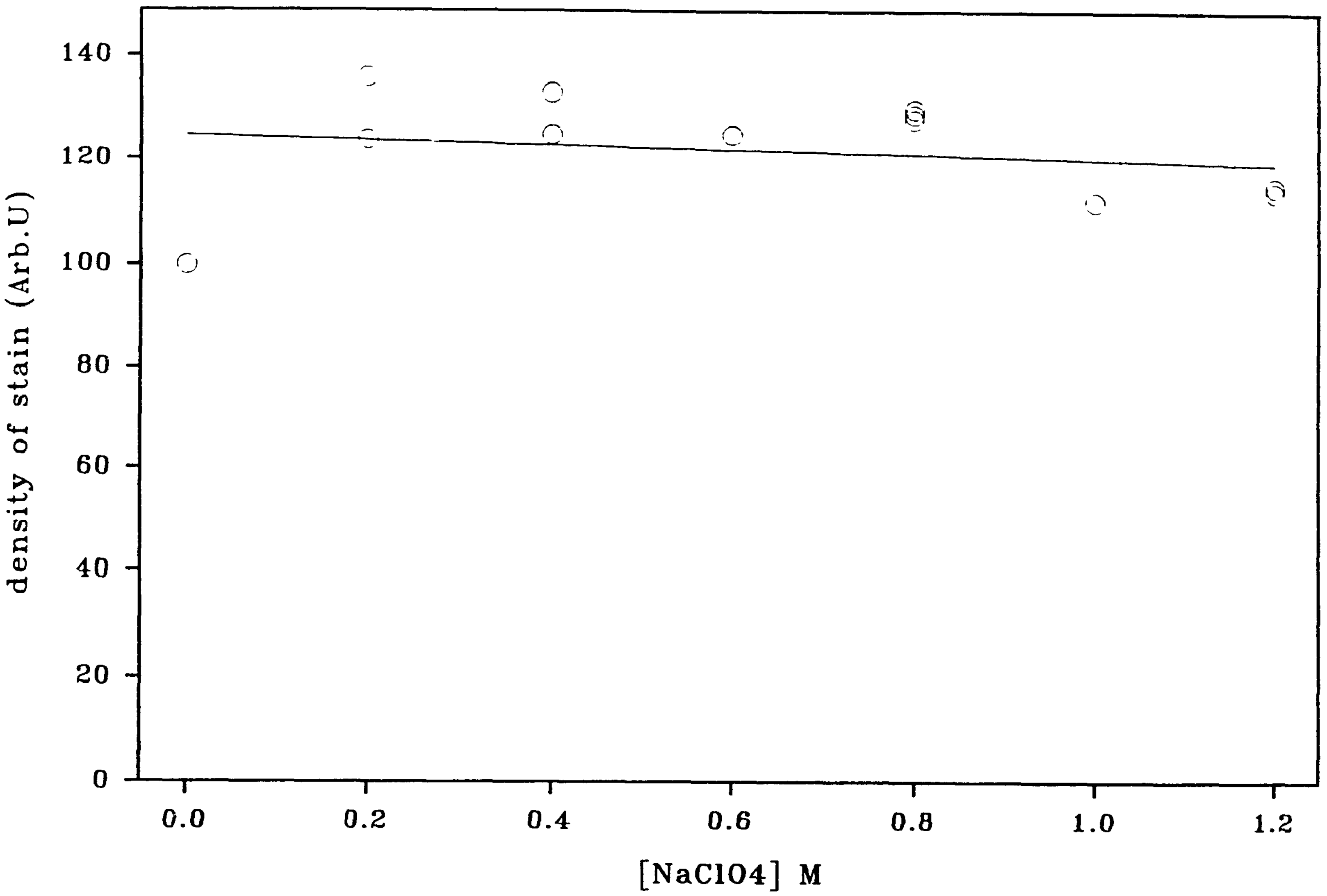
% dimeric enzyme
120
100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
[NaClO4] M

Figure 16: Proportion of crosslinked dimer to the concentration of $NaClO_4$ in presence of 1mM PGA.

[dansyl-$\beta\beta$] = 0.3μM

[$NaClO_4$] = 0.2 - 1.2M

Note: 100% is dansyl-$\beta\beta$ in MTM buffer (no $NaClO_4$)


140
120
100
80
60
40
20
0
density of stain (Arb.U)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
[NaClO4] M

interactions play a major role in determining the tertiary and quaternary structure of the protein (9,11). Once the hydrophobic interactions are disrupted, dissociation and inactivation may occur.

Brewer et al. (15, 47) have studied the dissociation of yeast enolase and observed that 1M potassium chloride facilitates dissociation while 1M potassium acetate does not; they also suggested that electrostatic or hydrogen bond interactions are not involved in the maintenance of the dimeric structure. Moreover, Brewer and coworkers suggest that hydrophobic interactions are responsible for subunit association.

C.5.0: Comparison of inactivation and dissociation.

As has been shown previously, incubation of rabbit muscle enolase in $NaClO_4$ results in both loss of activity (Fig. 4) and dissociation (Fig. 14). In order to study the relationship between inactivation and dissociation and to predict a model of inactivation and dissociation, it is necessary to be able to monitor the two processes quantitatively, under the same conditions. Since $NaClO_4$ also induces a dramatic increase of the fluorescence intensity of dansyl-$\beta\beta$, it is necessary to find out whether these changes reflect inactivation, dissociation or neither. For purposes of comparison the scale of the increase in fluorescence intensity of dansyl-$\beta\beta$ was inverted to 100-0 scale by using the end point of the change in the fluorescence equal to zero.

C:5.1: Time and [$NaClO_4$] dependence of inactivation and dissociation of enolase.

Enolase (300 nM) was incubated in 0.4M $NaClO_4$ for various times. The activity, the dissociation and the changes in the fluorescence intensity of dansyl-$\beta\beta$ were measured. A comparison of the rates of these processes (time courses shown in Figure 17) indicates that inactivation is faster than dissociation. Furthermore, At 0.5M $NaClO_4$, for example, 50% activity is lost within 10 minutes, but during same time, only 20% dissociation (Figure 18). At low concentrations of $NaClO_4$ (such as 0.2M) there are not many changes in inactivation and dissociation. We can conclude that inactivation occurs prior to dissociation; $NaClO_4$ inactivates the dimeric enzyme and causes dissociation.

It has been mentioned previously that $NaClO_4$ induces dissociation and inactivation of the $\gamma\gamma$ isozyme. Trepanier et al. used polarization of the fluorescein group as a probe for dissociation of FITC-$\gamma\gamma$. By comparing of the rates of the two processes, it was concluded that dissociation occurs before inactivation (6). Is the difference between the effects of $NaClO_4$ on dissociation and inactivation of the $\gamma\gamma$ and $\beta\beta$ isozymes due to different techniques of dissociation monitoring?

In order to test this the $\gamma\gamma$ isozyme (0.3 μM) was incubated in 0.4M $NaClO_4$ and aliquots were removed at varying times. These were then applied to the usual activity assay, crosslinking and SDS-PAGE experiments, using the same conditions

Figure 17: Time dependence of inactivation, dissociation and fluorescence intensity of dansyl-$\beta\beta$.

[dansyl-$\beta\beta$] = 0.3μM

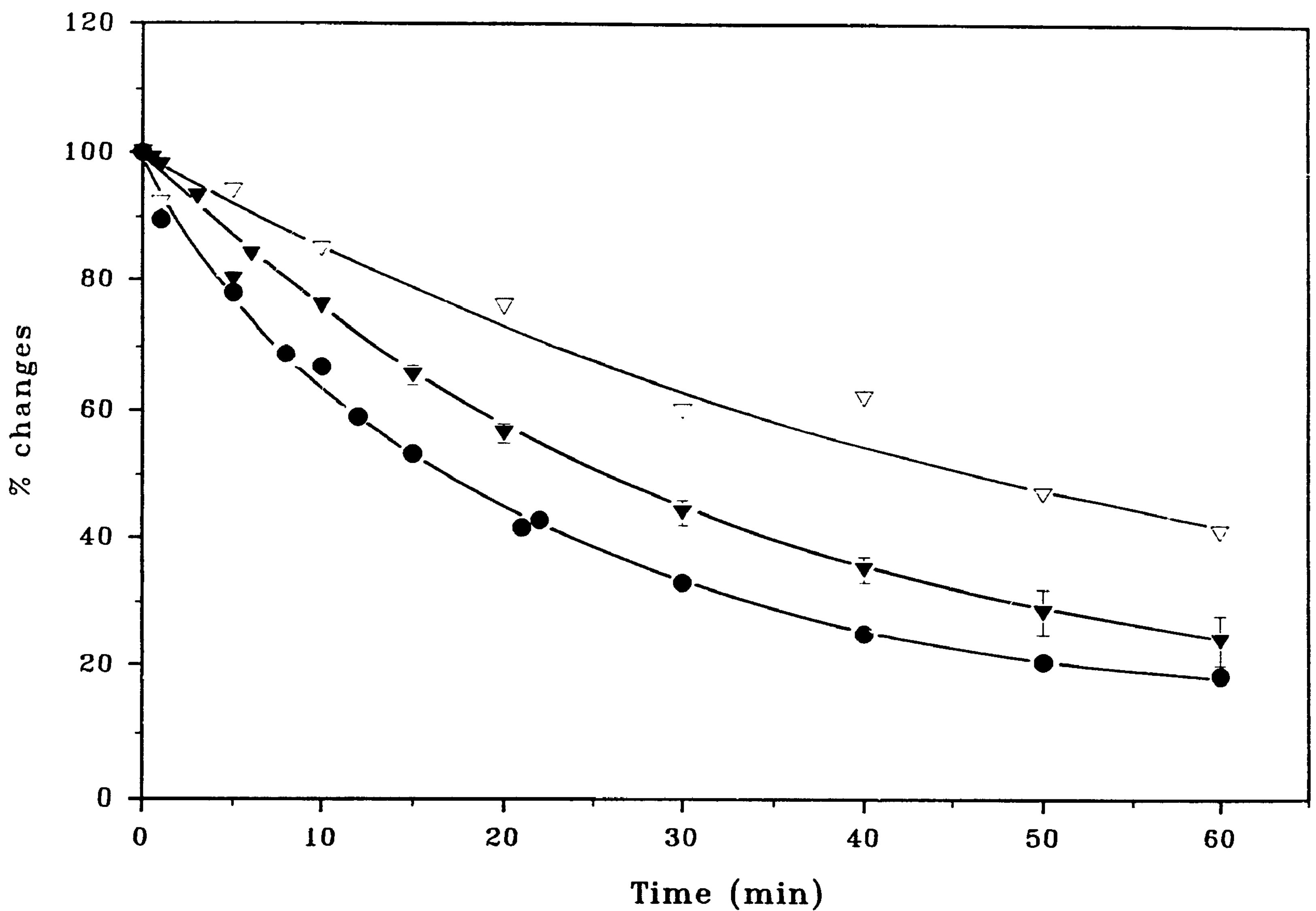
[$NaClO_4$] = 0.4M

● = Activity

▽ = % dimeric enzyme

▼ = Fluorescence intensity

The data (% dimeric enzyme) were replotted from Figure 13. The scale of fluorescence was inverted to 100-0 scale.



120
100
80
60
40
20
0
% changes
0
10
20
30
40
50
60
Time (min)

Figure 18: [$NaClO_4$] dependence of inactivation, dissociation and fluorescence intensity of dansyl-$\beta\beta$.

[dansyl-$\beta\beta$] = 0.3uM

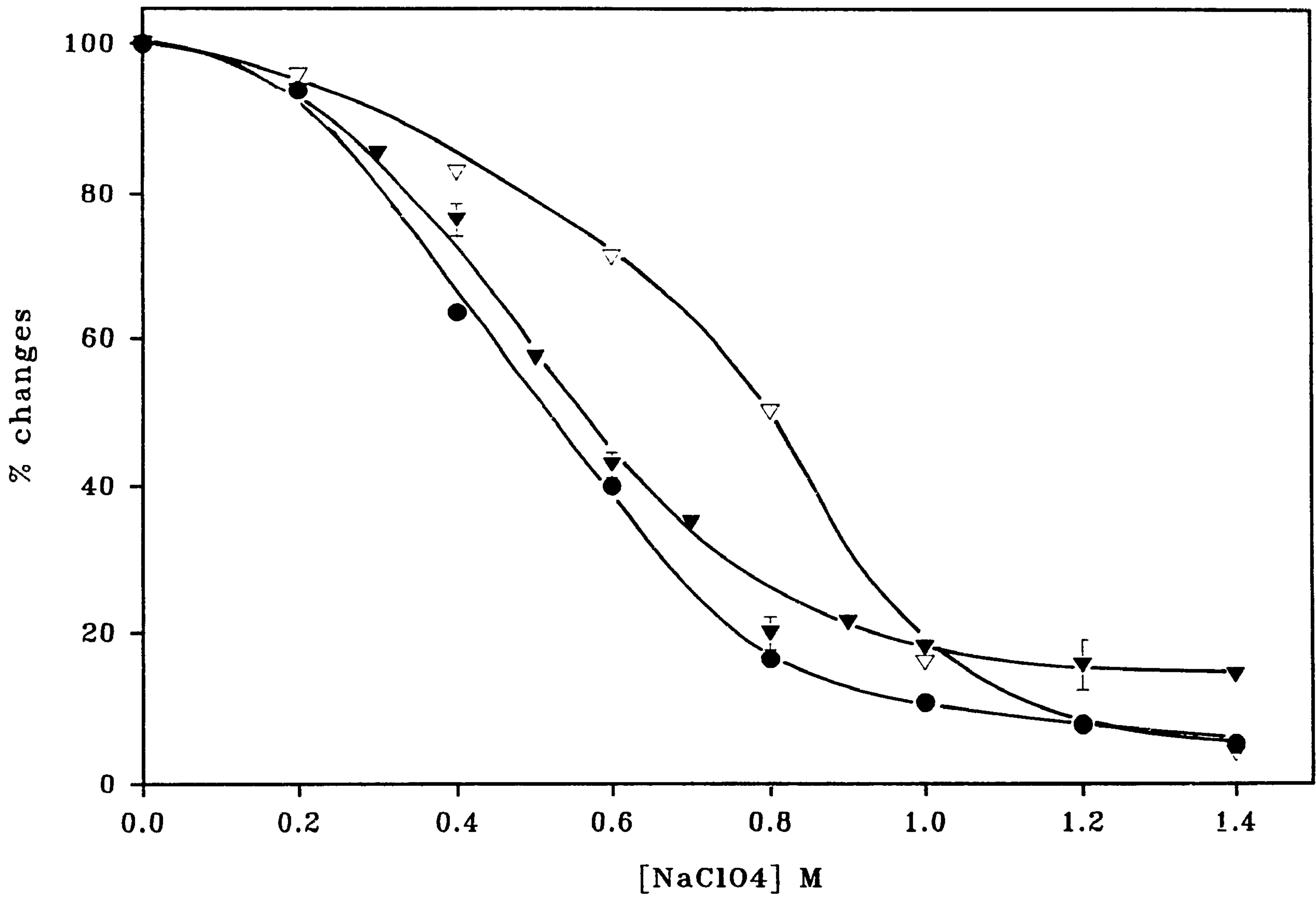
incubation time = 10 minutes

● = Activity

▽ = % dimeric enzyme

▼ = Fluorescence intensity

Note: the data (% dimeric enzyme) were replotted from Figure 14.The scale of fluorescence was inverted to 100-0 scale.



100
80
60
40
20
0
% changes
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
[NaClO4] M

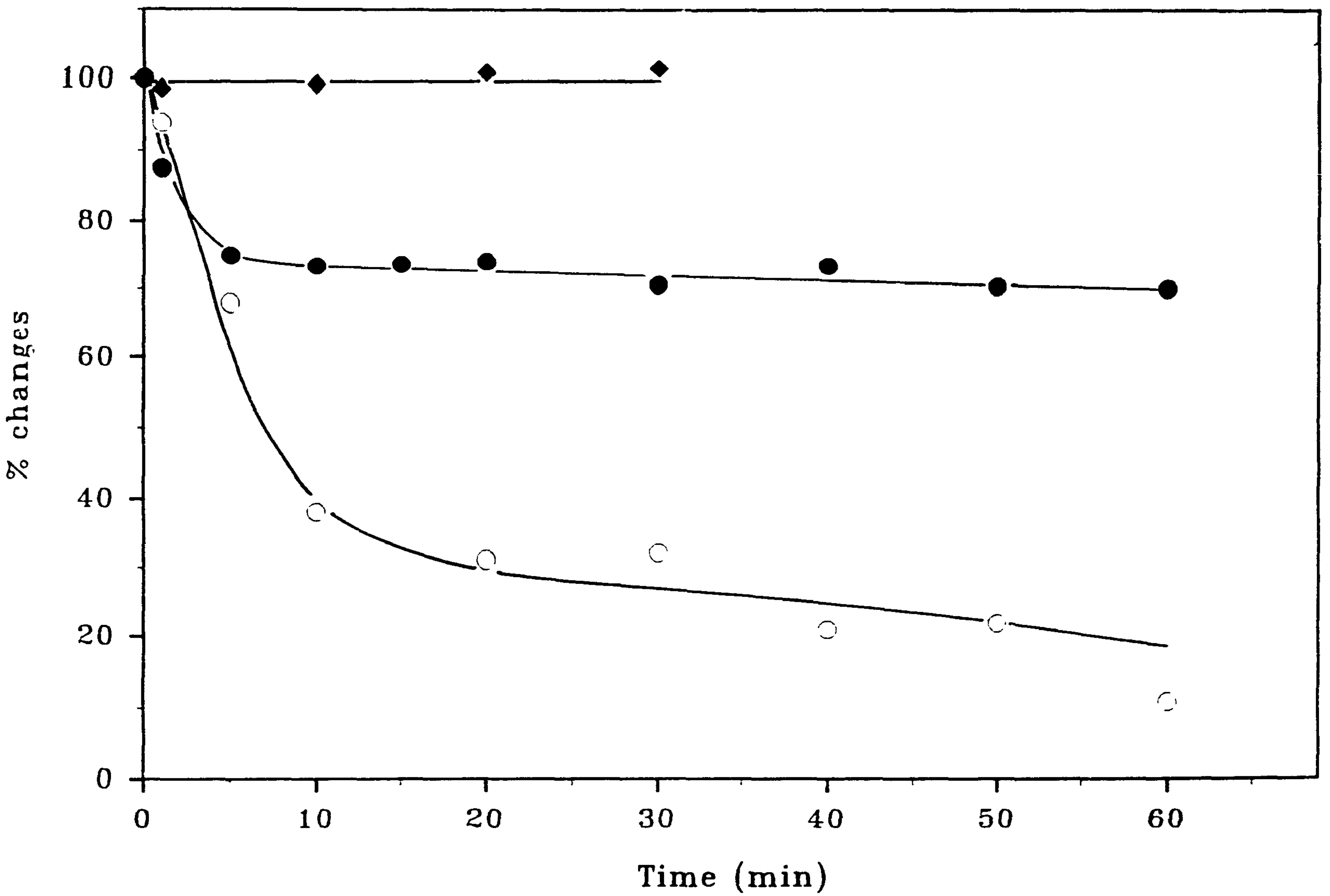
as for the $\beta\beta$ isozyme. The data for both inactivation and dissociation of $\gamma\gamma$ enolase are plotted in Figure 19. The results show that dissociation is faster than inactivation; similar results were obtained by Trepanier (6). Although the $\gamma\gamma$ and $\beta\beta$ isozyme have about 80% sequence identity (9,13), it may be that the differences in susceptibility of the $\gamma\gamma$ and $\beta\beta$ isozymes to inactivation and dissociation by $NaClO_4$ are due to differences in structure rather than the type of technique used.

It is clear that changes in the fluorescence intensity of dansyl-$\beta\beta$ in $NaClO_4$ does not follow the dissociation rate which is clear in the time and [$NaClO_4$] dependence graphs (Fig. 17 and 18). Thus, it can be concluded that the changes in fluorescence intensity of dansyl-$\beta\beta$ do not probe dissociation. It appears that the changes in fluorescence intensity follow inactivation to approximately a (10%) degree of difference. Since the 10% degree of difference between inactivation and fluorescence intensity changes appears in both Figures 17 and 18, and the data were duplicated, this indicates that the changes in fluorescence intensity do not monitor the inactivation. This observation could be explained by complex factors such as conformational changes in the tertiary and quaternary structures which would cause inactivation and dissociation and consequently affect the probe. This can be confirmed by preventing the dissociation and looking at the effects of $NaClO_4$ on inactivation and fluorescence intensity of dansyl-$\beta\beta$.

Figure 19: Inactivation and dissociation of the $\gamma\gamma$ isozyme in 0.4M $NaClO_4$.

[$\gamma\gamma$]	=	0.3uM
◆	=	Activity in 0.4M NaCl
●	=	Activity in 0.4M $NaClO_4$
○	=	% dimeric enzyme

The experiment of method applied to determine the % of dimeric enzyme of $\gamma\gamma$ enolase was the same as for $\beta\beta$.



% changes
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (min)

C:5.2: Time and [$NaClO_4$] dependence of inactivation (dissociation prevented).

In order to further understand the relationship between inactivation and dissociation of dansyl-$\beta\beta$, the effects of sodium perchlorate on enzymatic activity where be investigated under conditions dissociation was prevented. This approach answered the question: "does inactivation always occur via dissociation?"

Dissociation can be prevented by covalently crosslinking the enzyme with BS^3 (prior to incubation in salt) or by addition of substrate (as in Figure 9). The efficiency of crosslinking was about 90% (by SDS-PAGE, data not shown), with specific activity at about 90% of the original enzyme. The inactivation of the crosslinked enzyme, and the non-crosslinked enzyme in the presence of PGA, are shown in Figures 20 and 21. Both treatments greatly decrease inactivation at low concentrations of $NaClO_4$ (up to 0.4M). At high concentrations (>1.0M), treatment with PGA prevented about 50% of inactivation; crosslinking provided almost no protection against inactivation.

The stability against inactivation of the non-crosslinked enzyme with PGA is greater than that of the crosslinked enzyme. Figure 20, ∇, shows that the addition of PGA after 12 minutes of enzyme incubation in $NaClO_4$ does not reverse the loss of activity. So, the enzyme was incubated in $NaClO_4$ for one hour; PGA was then added and the mixture was left for a further hour. Then, an aliquot was removed, crosslinked and subjected to SDS-PAGE: no crosslinked dimer was observed (Figure 22, lane 3). These results indicate that the effects of PGA on the stability of the

Figure 20: Inactivation of the native enolase with PGA and crosslinked enzyme in 0.4M $NaClO_4$ as a function of incubation time.

Incubation buffer = MTM buffer + 0.4M $NaClO_4$, (unless otherwise stated)

[$\beta\beta$]	=	0.3μM
●	=	dansyl-$\beta\beta$
▼	=	dansyl-$\beta\beta$ (+ 1mM PGA in incubation buffer)
▽	=	dansyl-$\beta\beta$ (1mM PGA added to incubation buffer after 12 minutes of enzyme addition)
○	=	$\beta\beta$-x (crosslinked enzyme in MTM buffer + 0.4M NaCl)
□	=	$\beta\beta$-x (crosslinked enzyme)

All data were corrected for the inhibitory effects of Na^+ or the presence of PGA in the incubation buffer.

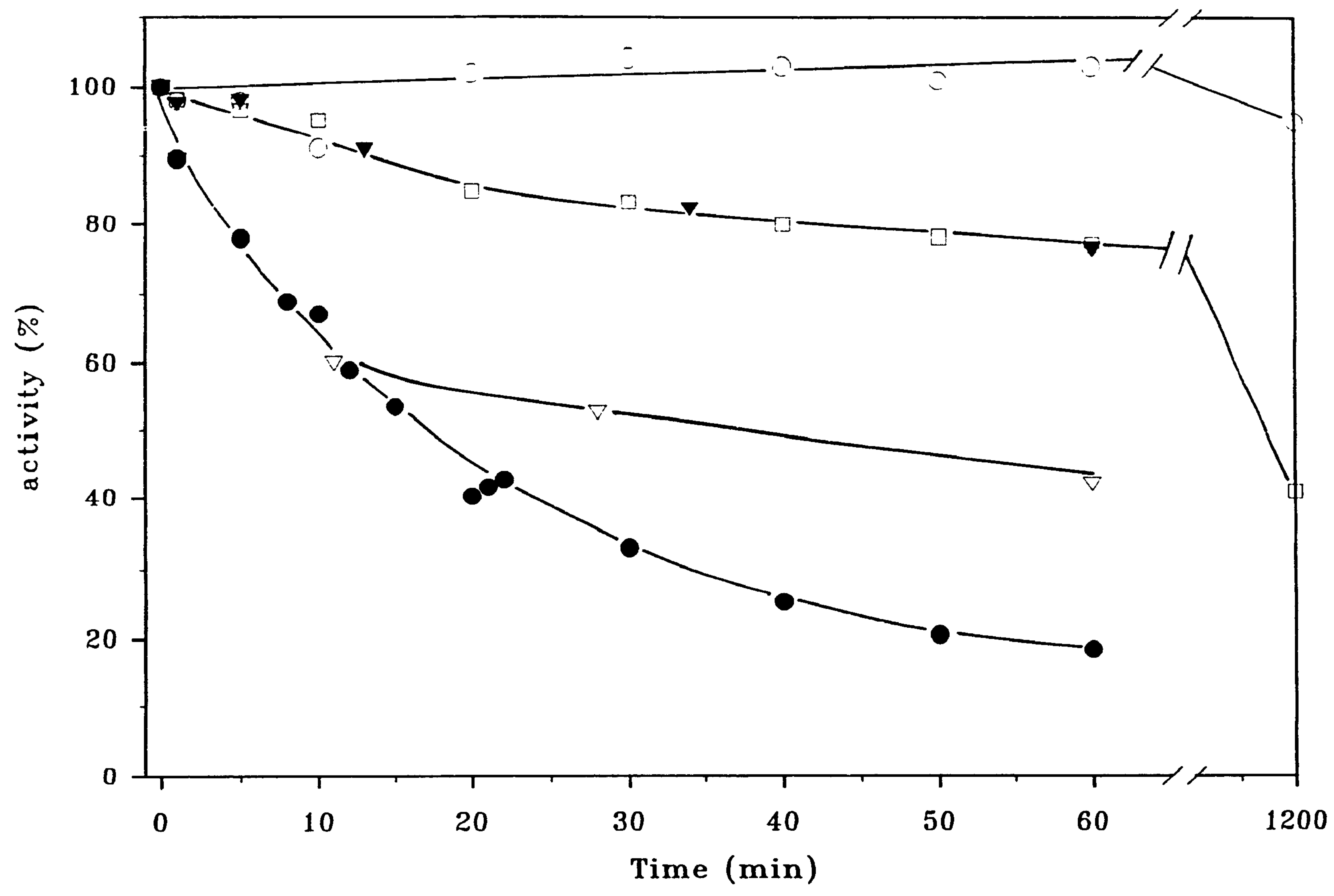

activity (%)
0
20
40
60
80
100
0
10
20
30
40
50
60
1200
Time (min)

Figure 21: Inactivation of the native enolase with PGA and crosslinked enzyme as a function of [$NaClO_4$].

Incubation buffer = MTM buffer + various of [$NaClO_4$]

Incubation time = 10 minutes

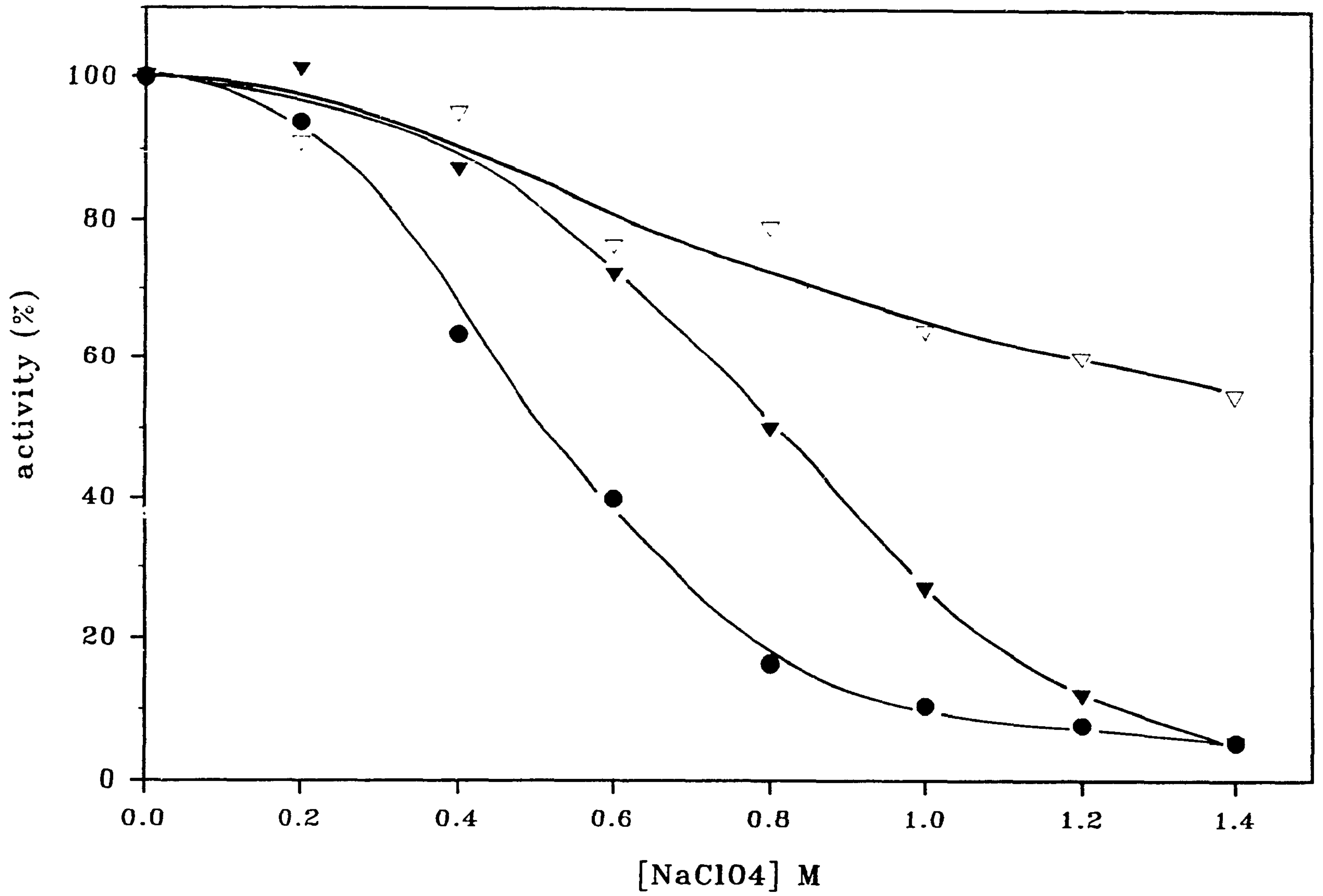
[$\beta\beta$] = 0.3uM

● = dansyl-$\beta\beta$

▿ = dansyl-$\beta\beta$ (+1 mM PGA in incubation buffer)

▾ = $\beta\beta$-x (crosslinked enzyme)

All data were corrected for the inhibitory effects of Na^+ and the presence of PGA in the incubation buffer.



activity (%)
100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
[NaClO4] M

Figure 22: Lack of reassociation in the presence of PGA and $NaClO_4$ detected by crosslinking and SDS-PAGE.

$[\beta\beta]$ = 90nM

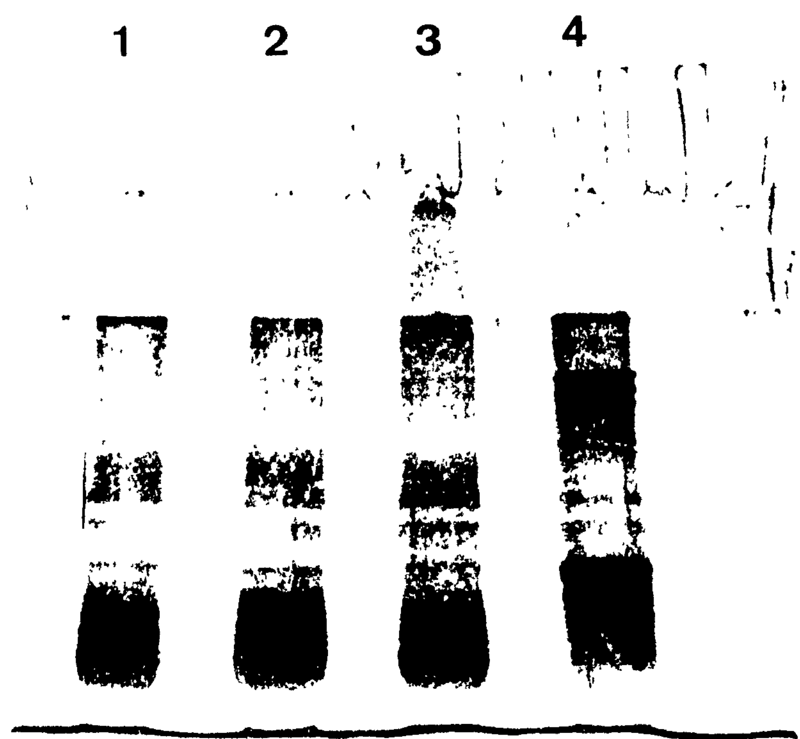
$[NaClO_4]$ = 0.8M

lane 1 = $\beta\beta$ incubated in $NaClO_4$ for 1 hour then crosslinked with glutaraldehyde

lane 2 = as in lane 1, PGA was added with glutaraldehyde

lane 3 = $\beta\beta$ was incubated in $NaClO_4$ for 1 hour; then 1mM PGA was added to the mixture and left for a further hour and finally crosslinked with glutaraldehyde

lane 4 = the glutaraldehyde was added one or two minutes following incubation of $\beta\beta$ in $NaClO_4$


1
2
3
4

enzyme are due neither to reactivation nor reassociation. Similar results of the effects of PGA on reactivation and reassociation of the $\gamma\gamma$ isozyme have been reported by Trepanier et al. (6) and attributed to a local effect on the structure of the enzyme. Above all, the results shown in Figures 20 and 21 indicate that the inactivation that is occurring in both treatments is due to inactivation of the dimeric enzyme. These results also confirm that inactivation by $NaClO_4$ can occur in the absence of dissociation. The next section will focus on the effects of $NaClO_4$ on fluorescence intensity of dansyl-$\beta\beta$ when dissociation is prevented by substrate addition or covalently crosslinked with BS^3.

C:5.3: Fluorescence intensity of dansyl-$\beta\beta$ in $NaClO_4$ (dissociation prevented)

The previous suggestion (C:5.1) in regard to the relationship of fluorescence intensity changes of dansyl-$\beta\beta$ to the inactivation and dissociation was also investigated. The crosslinked enzyme (dansyl-$\beta\beta$-x) and the native enzyme with PGA were incubated with $NaClO_4$. Figure 23 shows that substrate addition prior to incubation in $NaClO_4$ almost prevents the effects on the fluorescence intensity observed in the absence of substrate The crosslinked enzyme exhibited greater than 20% change in the fluorescence intensity after 60 minutes incubation in 0.4M $NaClO_4$ even though the enzyme is in a dimeric form. These results confirm that conformational changes occurred on both the tertiary and quaternary structure of the enzyme during incubation in $NaClO_4$. Moreover, the fluorescence intensity changes of dansyl-$\beta\beta$ do not probe inactivation since the enzyme incubated with 0.4M $NaClO_4$

enzyme incubated with 0.4M $NaClO_4$ in the presence of PGA, lost 20% of its activity and had no changes in the fluorescence intensity.

Figure 23: Fluorescence intensity of dansyl-$\beta\beta$ with PGA and dansyl-$\beta\beta$-x (the crosslinked enzyme) in $NaClO_4$

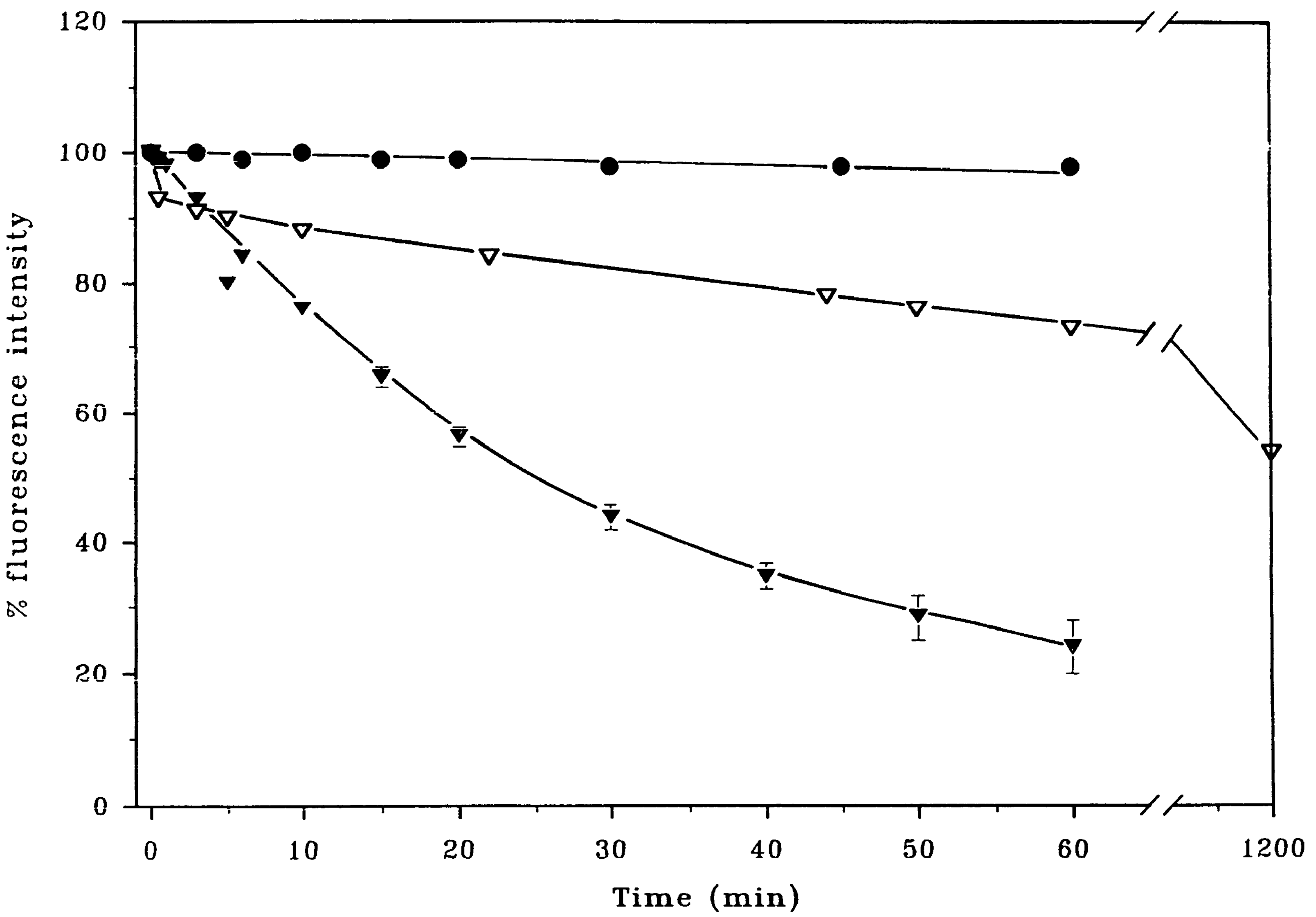
[$\beta\beta$] = 0.3mM

● = dansyl-$\beta\beta$ in MTM buffer + 1mM PGA and 0.4M $NaClO_4$

▿ = dansyl-$\beta\beta$-X in MTM buffer + 0.4M $NaClO_4$

▾ = dansyl-$\beta\beta$ in MTM buffer + 0.4M $NaClO_4$

The scale of fluorescence was inverted to 100-0 scale.

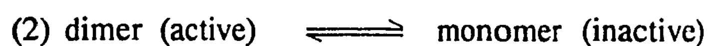


120
100
80
60
40
20
0
% fluorescence intensity
0
10
20
30
40
50
60
1200
Time (min)

CONCLUSION

The dimeric enzyme, enolase, exists in at least three isozymes ($\alpha\alpha$, $\beta\beta$, $\gamma\gamma$), with similar sequences in mammalian tissues. Enolase isozymes can be inactivated and dissociated by agents such as pressure or chaotropic salts; the different isozymes differ in sensitivity to these agents. $NaClO_4$ inactivates and dissociates the $\beta\beta$ isozyme of enolase; three models could explain the result of inactivation:

(1) dimer (active) $\rightleftharpoons$ dimer (inactive) $\rightleftharpoons$ monomer (inactive)



(2) dimer (active) $\rightleftharpoons$ monomer (inactive)

(3) dimer (active) $\rightleftharpoons$ monomer (active) $\rightleftharpoons$ monomer (inactive)

In order to understand the relationship between quaternary structure and catalytic activity, the effects of $NaClO_4$ and NaCl on both enzymatic activity and dissociation of rabbit enolase $\beta\beta$ isozyme were studied. The $\beta\beta$ enolase was covalently labelled with dansyl chloride in order to probe the conformational changes that may occur during inactivation and dissociation. To monitor dissociation, the enzyme was crosslinked with glutaraldehyde and then analyzed by SDS-PAGE and visualized by silver stain. Enolase in 0.6M NaCl could be crosslinked, but enzyme in 0.6M $NaClO_4$ could not, indicating dissociation. The dissociation could be prevented before or during the incubation in $NaClO_4$ by addition of glutaraldehyde or the substrate which allow for the detection of the dimer at any time during incubation. Thus, we found that crosslinking and SDS-PAGE is a very useful technique for detecting and quantitatively measuring the dissociation rate of $\beta\beta$ enolase. The relative proportions of the decreases in staining intensity of the dimer

bands (as determined by densitometry of the stained gel) were measured as a function of the length of incubation in salts or salt concentration.

Enolase requires 0.8M $NaClO_4$ to reach the inactivation equilibrium. Inactivation and dissociation are time and [$NaClO_4$] dependent; NaCl has no effect on these processes. However Na^+ inhibition occurred and was corrected for in all experiments.

Sodium perchlorate is one of the chaotropic salts; it increases the lipophilicity of water and weakens the hydrophobic bonds of the enzyme. Consequently, inactivation and dissociation may occur. The inactivation with $NaClO_4$ mostly accompanies conformational changes in tertiary and/or quaternary structure. These changes might be partially irreversible for some enzymes. The $\beta\beta$ isozyme recovers about 60% of its activity after salt removal, and the loss could be due to the enzyme not renaturing to its original conformation.

A decrease in intensity and red shift in the tryptophan fluorescence emission spectrum were observed during incubation of the enzyme in $NaClO_4$. Moreover, exposure of the labelled enolase (dansyl-$\beta\beta$) to $NaClO_4$ resulted in a dramatic increase in fluorescence intensity. This increase in fluorescence intensity of dansyl-$\beta\beta$ was also time and [$NaClO_4$] dependent. These results confirm an actual conformational change within the enzyme occurred as a result of exposure to $NaClO_4$.

At this point, since it was possible to monitor inactivation and dissociation under the same conditions, the relationship between the two processes was studied.

Inactivation was found to be faster than dissociation. At 0.5M $NaClO_4$, for example, 50% activity is lost within 10 minutes, but during same time, only 20% dissociation. Thus we can conclude that inactivation occurs prior to dissociation; $NaClO_4$ inactivates the dimeric enzyme and causes dissociation.

The above conclusion was confirmed by inactivation of $\beta\beta$ enolase by $NaClO_4$ in which dissociation was prevented. Dissociation was prevented by covalently crosslinking the enzyme with bis (sulfosuccinimidyl) suberate (BS^3) or by adding the substrate PGA. Table I summarizes the effects of PGA and covalent crosslinking of $\beta\beta$ enolase on dissociation, inactivation and fluorescence intensity of dansyl-$\beta\beta$. Both treatments greatly decreased the rate of inactivation at low concentration of $NaClO_4$ (up to 0.4M); at high concentration of $NaClO_4$ ($>$ 1.0M),crosslinking provided almost no protection against inactivation.

The changes in the fluorescence intensity of dansyl-$\beta\beta$ in $NaClO_4$ neither exactly follow inactivation nor dissociation rates. This could be due to complex conformational changes in tertiary and quaternary structure which could cause inactivation and dissociation and consequently affect the probe. $NaClO_4$ causes a conformational change on the crosslinked enzyme or the native enzyme in the presence of the substrate; this is reflected by an increase in the fluorescence intensity of dansyl-$\beta\beta$ in the dimeric form.

In summary, we can conclude that inactivation by $NaClO_4$ can occur in the absence of dissociation and that the observed effects of $NaClO_4$ on rabbit muscle

enolase are due to a partial inactivation of the dimeric enzyme and to a dissociation into inactive monomers (model 1).

Table I

THE EFFECTS OF PGA AND COVALENT CROSSLINKING OF ENOLASE ON DISSOCIATION, INACTIVATION AND FLUORESCENCE INTENSITY OF DANSYL-$\beta\beta$

Incubation	low [$NaClO_4$], 0.4M 60 min		high [$NaClO_4$], 1.4M 10 min	
Processes	PGA	Crosslinking	PGA	Crosslinking
Dissociation	prevent	prevent	prevent	prevent
Inactivation	20%	20% (20 h.,41%)	46%	95%
Fluorescence Intensity	no significant change	20% (20 h.,54%)	20%	80%

COMMENT AND FURTHFR WORK:

Since the inactivation of $\beta\beta$ enolase by Na(..)$_4$ precedes dissociation and can occur without it, we expect that it produces inactive monomers. However, we don't have evidence for the presence of inactive monomers of the $\beta\beta$ isozyme in the absence of $NaClO_4$.

The low recovery of activity had not permitted us to measure the rate of reassociation of enolase. Optimization of the reactivation conditions to get full recovery of activity would be a recommended step to further studies on the relationship between inactivation and dissociation. It would be interesting to investigate the likelihood of a fully reversible ßß enolase inactivation in order to: (1) determine whether the monomer is inactive in the absence of the agents inducing inactivation and dissociation; (2) see whether conformational changes, necessary for the active conformation, occur as a result of subunit interaction. Under mild conditions, enolase might be immobilized and then dissociated with $NaClO_4$. The enzymatic studies on the immobilized monomer should be performed to learn how the monomer differs catalytically from the dimer.

A study of the effects of both ionic strength and pH on the dimer-monomer equilibrium is recommended in order to learn about the nature of the forces involved in subunit interactions and stability, and whether ion pairs contribute to subunit interaction in mammalian enolase.

The addition of PGA to $\beta\beta$ enolase provided more resistance to inactivation by $NaClO_4$ than covalent crosslinking. The role of PGA in providing protection of the enzyme against dissociation and partial inactivation is not fully understood. The addition

of substrate increases the fluorescence of $\beta\beta$ by 30% but does not affect the fluorescence of dansyl-$\beta\beta$ in the MTM buffer. Nevertheless, the substrate prevents the $NaClO_4$-induced increase in the fluorescence intensity of dansyl-$\beta\beta$. Addition of substrate or tartronate semialdehyde phosphate, a tight binding inhibitor to the native or labelled enolase under varying conditions of temperature or pressure may help in understanding the role of the substrate.

The effects of $NaClO_4$ on the mammalian $\gamma\gamma$ and $\beta\beta$ enolases have been extensively studied. However, $\alpha\alpha$ enolase has not been examined using $NaClO_4$. Since crosslinking followed by SDS-PAGE is a useful technique to monitor dissociations, comparative studies of inactivation and dissociation of the three isozymes under the same conditions using this technique may provide new clues.

Site-directed mutagenesis of $\beta\beta$ enolase near the subunit contacts and the tryptophan residues may help in understanding the subunit interactions. A number of experiments on inactivation, dissociation and fluorescence of native or mutant enolase would be interesting to investigate.

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