Correlation between Activity and Dynamics of the Protein Matrix of Phosphorylase *b*†

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ABSTRACT: Quenching of the tryptophan fluorescence of phosphorylase *b* was studied by using iodide and acrylamide. Steady-state measurements indicated that all indole side chains were accessible to the nonionic quencher, although only 3 out of the total of 12 residues could be quenched by I^-. From Stern–Volmer plots and the fluorescence lifetime data, it was concluded that the quenching was mainly of dynamic character. The value of the collisional quenching rate constant was found to be an order of magnitude less than that obtained in the case of fully exposed tryptophans. The relatively high activation energy, 30.9 kJ/mol, of the diffusion-controlled process and the value of the activation entropy suggest that the diffusion takes place in a fluctuating, structured medium. In spite of the application of sensitive fluorescent techniques, no gross conformational changes were found in the presence of acrylamide. However, the catalytic rate of the glycogen synthesis was decreased with the residual activity of the enzyme, proportional to the concentration of the probe. Binding of activator (AMP) and substrates (glucose 1-phosphate and glycogen) was found to be unaffected by acrylamide in concentrations applied (0–0.8 M). In a similar manner, activation enthalpy did not change in the presence of the quencher either. The complete reversibility of both activity inhibition and fluorescence quenching ruled out the irreversible denaturation of the enzyme or the covalent modification of any of the functional groups. We concluded that a model, suggesting the cross-correlation of activity and fluctuation, was consistent with the experimental findings.

We know relatively little about the nature and function of motions in protein structures. The significance of the relatively large amplitude and "slow" (i.e., nanosecond time range) fluctuations is far from being completely understood (McCammon et al., 1979; Gurd & Rothgeb, 1979). The early experimental recognition by Linderstrøm-Lang of the fact that proteins fluctuate was followed by a series of theoretical and experimental studies (Linderstrøm-Lang & Schellman, 1959). Green & Ji (1972) emphasized the importance of thermal energy coupling between environment and proteins and elaborated the electro–mechano–chemical energy transducing theory. Careri's (1974) statistical thermodynamic approach, the piezoelectric model proposed by Caserta & Cervigni (1974), let alone the work of Low & Somero (1975), stressed the importance of the hydration energy and exploited the "slow" large-scale fluctuations of the proteins in order to explain the energetic background of the enzyme activity; all revealed different aspects of the same problem, i.e., the interaction between solvent and protein motion and the role of protein fluctuation in the enzyme action (Careri, 1974; Careri et al., 1979; Caserta & Cervigni, 1974; Low & Somero, 1975).

The molecular enzyme kinetic model also relied on the conception of the protein fluctuation and solvent interaction by using manifest physical parameters like environmental microviscosity, mass distribution of the solvent molecules, and collisional frequency of the environmental and protein particles (Somogyi & Damjanovich, 1971, 1975; Damjanovich & Somogyi, 1973, 1978; Somogyi et al., 1978).

It is customary to distinguish between two classes of conformational fluctuation. The first one is the small amplitude oscillation of relative distances between the various parts of proteins. Suezaki & Go (1975) described this type of fluctuation. More frequently considered are those fluctuations where a given structural part of the protein is randomly destructed, and a new configuration is built up. These large-scale fluctuations were also extensively studied by Eftink & Ghiron (1975, 1977), Lakowicz & Weber (1973), Saviotti & Galley (1974), and Frauenfelder's team (Austin et al., 1975; Alberding et al., 1978; Frauenfelder et al., 1979).

Changes in the enzyme catalysis and regulation were extensively studied while the environmental viscosity was increased and by assuming that the latter must impair the protein fluctuation (e.g., Gavish & Werber, 1979; Damjanovich et al., 1972; Trón et al., 1976; Jancsik et al., 1975; Laurent & Öbrink, 1972). The significance of the large amplitude fluctuations, however, was firmly established only in the case of the ligand binding of lysozyme and myoglobin (Nakanishi et al., 1972, 1973; Frauenfelder et al., 1979).

To our knowledge, no experimental attempt aimed at studying fluctuation and catalysis in the same protein simultaneously has been made. In our experiment, acrylamide was applied to quench the tryptophan fluorescence of phosphorylase *b* enzyme. Activity tests revealed that acrylamide significantly decreased the enzyme activity. Since both the quenching of fluorescence (i.e., a parameter indicating the protein fluctuation) and the decrease in the activity were caused by the same agent, the possibility of finding a correlation between the dynamic and functional properties of the enzyme seemed to be real.

Experimental Section

The glycogen phosphorylase *b* (EC 2.4.1.1) was prepared from rabbit muscle, following the method of Fischer & Krebs (1962) modified to the extent that 2-ME[1] was substituted for cysteine. For removal of the small molecules essential for crystallization, the four-times recrystallized enzyme was passed through a Sephadex G-25 column immediately before use (Kleppe & Damjanovich, 1969). The specific activity of the enzyme was found to be 56 units/mg as determined by the method of Illingworth & Cori (1953). With the use of a

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[1] Abbreviations used: 2-ME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid disodium salt; G-1-P, glucose 1-phosphate; Cl_3-AcOH, trichloroacetic acid.

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molecular weight of 185 000 (Seery et al., 1967) and $A^{1\%}_{280,1cm}$ = 13.2 (Buc-Caron & Buc, 1975), the molar concentration of the enzyme was calculated from the absorbance at 280 nm and expressed as that of the dimer.

Kinetic and fluorometric experiments as well as gel filtration were performed in 50 mM Tris-HCl buffer (pH 6.8) containing 10 mM 2-ME and 1.5 mM EDTA. The glycogen primer was prepared from rabbit liver by the Cl_3AcOH extraction method (Stetten et al., 1956).

The acrylamide of analytical grade was obtained from Serva and was further purified by recrystallization from acetone and chloroform. All other chemicals were of reagent grade.

Enzyme Assay. Enzyme activity was measured in the direction of glycogen synthesis at steady-state kinetic conditions. Assay mixtures contained 32 mM G-1-P, 1 mM AMP, 1% (w/v) glycogen, and 5×10^{-8} to 1×10^{-7} M enzyme, unless otherwise stated.

The initial rate of the enzyme reaction was calculated from the amount of inorganic phosphate liberated from G-1-P, determined by the method of Taussky & Schorr (1953). Data displayed as Lineweaver–Burk plots were fitted by the linear regression method.

Fluorescence Quenching Experiments. Steady-state fluorescence measurements were performed in a Hitachi Perkin–Elmer MPF-4 spectrophotofluorometer equipped with a thermostated cell holder. The experiments were carried out in 1 × 1 cm silica cells in a ratio-operating manner with the use of a 4-nm bandwidth. The tryptophan residues of the enzyme were selectively excited at a wavelength of 295 nm (Sober, 1970). The optical density of protein solutions was less than 0.05 at the excitation wavelength in all cases. The fluorescence of the enzyme was quenched by progressive addition of small aliquots of a concentrated (6 M) acrylamide stock solution.

The fluorescence intensity of the phosphorylase *b* samples measured in the presence of acrylamide was corrected according to Parker (1968), since the quencher has a molar extinction coefficient of about 0.23 at 295 nm.

The iodide quenching experiments were carried out by adding different quantities of 3 M KI according to Honikel & Madsen (1973).

For analysis of quenching, the Stern–Volmer equation, $F_0/F = 1 + K_{sv}[Q]$, was applied, where F_0 is the unquenched fluorescence, F is the quenched fluorescence, K_{sv} is the Stern–Volmer constant (supposed to be $K_{sv} = k_q\tau_s$, where k_q correlates with the collisions of the quencher and τ_s = the unquenched singlet lifetime of the fluorophor), and $[Q]$ is the molar concentration of the quencher. Experimental data were fitted by the linear regression method.

Fluorescence Lifetime Measurements. Fluorescence lifetime measurements were carried out with time-correlated single-photon counting, using a nanosecond fluorescence spectrometer built in our laboratory. The data were analyzed by a computer program based on the method of nonlinear least squares, written by Dr. J. Szőke (Central Physical Research Institute, Budapest).

Results

Quenching of Phosphorylase b Fluorescence. The fluorescence of phosphorylase *b* was quenched by acrylamide, and the emission spectra were structured as seen in Figure 1. The small shoulder on the right side of the emission peak was probably due to tryptophyl side chains situated on, or near, the surface of the protein (Burstein et al., 1973). Increasing acrylamide concentrations did not cause any shift or change in the shape of the spectra. All this implies the absence of a gross, quencher-induced conformational change of the protein.

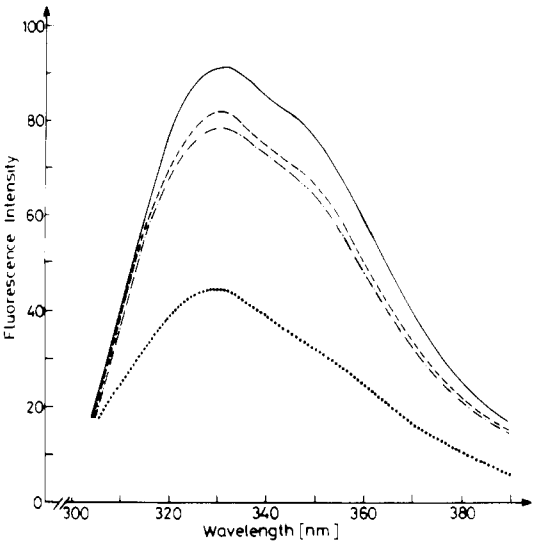

FIGURE 1: Fluorescence emission spectra of phosphorylase *b* at the following conditions: in the absence (—) and in the presence of 0.1 M (- - -) and 0.8 M (···) acrylamide at 30 °C, and in the presence of 0.1 M acrylamide (- · -) at 40 °C. Excitation wavelength, 295 nm; excitation and emission bandwidth, 4 nm.

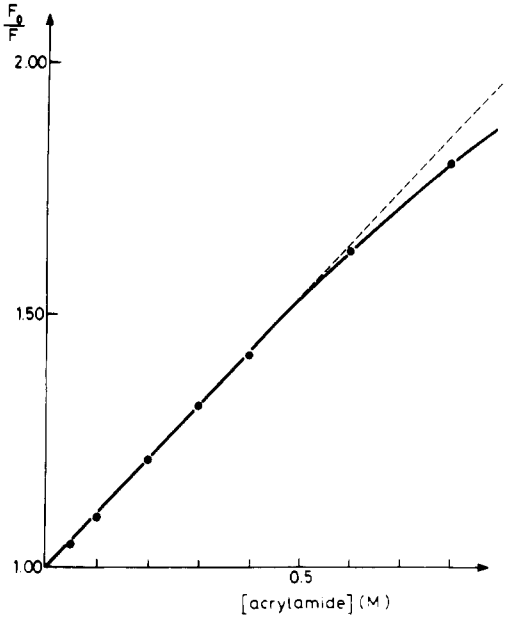

FIGURE 2: Stern–Volmer plot for the quenching of phosphorylase *b* fluorescence by acrylamide, at 30 °C. The excitation wavelength was 295 nm, and the emission was monitored at 333 nm.

The Stern–Volmer plot of acrylamide quenching was found to be linear until 0.5 M concentration of the quencher was reached (Figure 2). A slight downward curvature at higher acrylamide concentrations may be due to different classes of tryptophan residues in phosphorylase *b* with different collisional quenching constants. The results of iodide quenching experiments supported the idea that there existed different classes of tryptophan fluorophors in the enzyme. According to these results, only 3 tryptophans out of the total of 12 per monomer were exposed to the solvent.

The effective quenching constant (K_{sv}^{eff}) was calculated from the initial, linear part of the Stern–Volmer plot (Figure 2). The measured quenching constant was found to be 1.28 M^{-1} at 30 °C. The fluorescence decay curves showed one major component and another, almost negligible, component. The

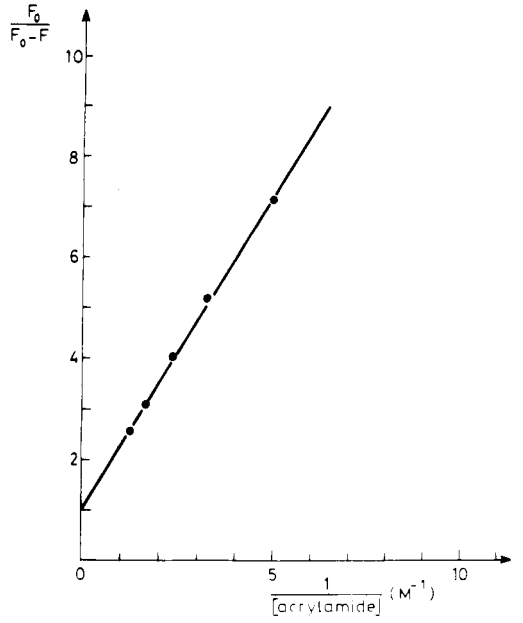

FIGURE 3: Modified Stern–Volmer plot for tryptophan fluorescence quenching of phosphorylase *b* by acrylamide. The concentration of the acrylamide varied from 0 to 0.8 M.

unquenched τ_s was 2.8 ns by using the major component, while in the presence of 0.4 M acrylamide it decreased to 1.8 ns. The rate constant (k_q) for the collisional encounter between tryptophan and acrylamide was found to be 4.8×10^8 M^{-1} s^{-1}. This value is an order of magnitude less than those obtained for model compounds with tryptophyl side chains fully exposed to the solvent [$(3–5) \times 10^9$ M^{-1} s^{-1}] (Eftink & Ghiron, 1976a). The number of fluorophors accessible to the quencher in different conditions was calculated by using the modified form of the Stern–Volmer plot (Lehrer, 1971). It was found that all tryptophyl side chains in phosphorylase *b* were accessible to acrylamide in all cases (Figure 3). These results provide straightforward evidence for the permeation of acrylamide molecules to the interior of phosphorylase *b*. The parallel drop in the lifetime accompanying the quenching suggests that the quenching process is mainly of dynamic character.

The temperature dependence of the quenching rate was studied between 20 and 40 °C in order to characterize the dynamic properties of phosphorylase *b* in terms of activation energy for the inward motion of acrylamide. The enzyme is not supposed to suffer any gross structural transition in this temperature range (Graves & Wang, 1972). Parallel quenching experiments showed that the fraction of accessible tryptophans for iodide was not changed significantly when the temperature was increased. The rate of collisional quenching at a given temperature was calculated from the K_{sv} and τ_s values. Estimation of temperature dependence of τ_s was done by assuming its proportionality to the fluorescence yield (Badley & Teale, 1969). The change of the latter was studied in the temperature range applied, through the decrease of the relative fluorescence intensity of the tryptophans on varying the temperature from 20 to 40 °C.

The Arrhenius plot of the acrylamide quenching (k_q vs. T^{-1}) proved to be linear. A value of 30.9 kJ/mol was obtained for the activation energy, which is twice as high as that for free tryptophan in water (Eftink & Ghiron, 1976a). This activation energy can be attributed to the fast rearrangements of weak hydrogen bonds and some van der Waals interactions in the local regions of the enzyme structure. These fast processes can be pictured as periodic formations of holes or "local cavities" in the enzyme matrix, i.e., certain types of fluctuations

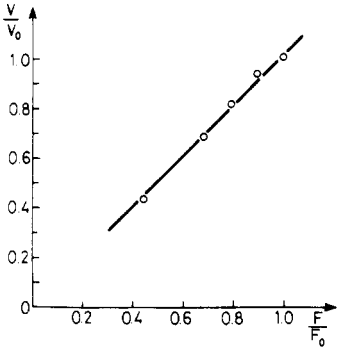

FIGURE 4: Relationship between the fractional decrease of enzyme activity and fluorescence emission measured in the same enzyme samples. The enzyme and the acrylamide were applied in concentrations of 0.1 mg/mL and from 0 to 0.8 M, respectively.

Table I: Recovery of Acrylamide-Inhibited Enzyme Activity upon Dilution[a]

sample	enzyme (μM)	acrylamide (M)	act. after dilution[b] (%)
A	0.6	0.2	100.0 ± 2.0
B	1.2	0.4	103.0 ± 2.5
C	1.8	0.6	103.0 ± 2.5
D	2.4	0.8	100.0 ± 2.0

[a] Samples containing enzyme and acrylamide in the concentrations indicated were incubated at 25 °C. After 5-min incubation time, each sample was diluted to the same final concentration (0.6 μM enzyme, 0.2 M acrylamide) by adding an appropriate volume of buffer. The diluted samples were thermostated at 30 °C. After 1 min, parallel aliquots were removed from enzyme activity determination. [Assay mixtures contained 0.2 M acrylamide, 32 mM G-1-P, 1 mM AMP, 1% (w/v) glycogen, and 1.5×10^{-7} M enzyme.] [b] The activity of sample A was taken as 100%. SD values were also indicated.

of the protein. The calculated value of activation entropy ($\Delta S^{\ddagger}$ = +25.1 J/K) seemed to support this idea. This value of activation entropy indicates an immediate increase in the disorder of the portions of the protein matrix surrounding the fluorophors. With the assumption that the collisional quenching rate is proportional to the frequency of the local structural fluctuations of the enzyme, it can be concluded that phosphorylase shows a nanosecond conformational fluctuation to a certain extent.

Effect of Acrylamide on the Activity of Phosphorylase b. While the concentration of the acrylamide was increased from 0 to 1 M, a monotonous decline in the enzyme activity was observed. The fractional decrease in enzyme activity and fluorescence intensity caused by acrylamide displayed a close linear correlation (Figure 4). The inhibition caused by acrylamide seemed to be completely reversible (Table I). Parallel fluorescence measurements of the same samples demonstrated that quenching of the fluorescence was also totally reversible. It should be noted that Eftink & Ghiron have also observed some 15% alteration in the activity of chymotrypsin and pepsin in the presence of 0.5 M acrylamide and a sign of slight denaturation of aldolase in the presence of high acrylamide concentration (Eftink & Ghiron, 1976b, 1977).

As shown in Figure 5, the acrylamide applied in concentrations up to 0.4 M did not influence the denaturation kinetics of the enzyme at 30 °C. At a high concentration of the quencher (1 M), exponential denaturation kinetics were observed. The immediate decrease in the activity level upon addition of the acrylamide cannot be attributed to the denaturation of the enzyme protein at practically any of the acrylamide concentrations applied. Even at a 1 M acrylamide

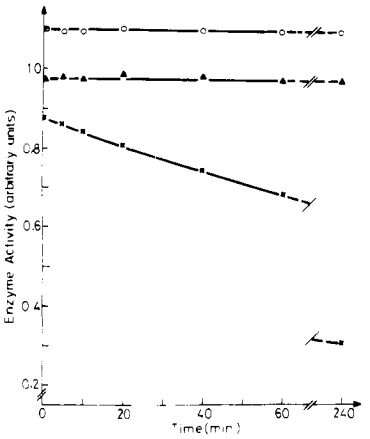

FIGURE 5: Denaturation kinetics of phosphorylase *b* measured at 30 °C. The activity of the enzyme is shown as a function of time. Incubation mixtures contained none (○), 0.4 M (▲), and 1 M (×) acrylamide and 0.1 mg/mL enzyme. From time to time, small aliquots (100 μL) were taken from the samples for activity measurements.

concentration, the denaturation could only slightly influence the activity assay due to the relatively short incubation time (5 min). Experiments were carried out in order to examine whether acrylamide had any influence on the binding parameters of the specific ligands responsible for the catalytic activity and the allosteric regulation of the enzyme. As can be seen in Figure 6A–C, the K_m constants of the ligands G-1-P, AMP, and glycogen did not change upon the addition of different concentrations of acrylamide, while a significant decrease was observed in the maximal rate (V_{max}) of the enzyme reaction.

Discussion

Lakowicz & Weber (1973) discovered that quenching of protein fluorescence by oxygen reported a periodical accessibility of the fluorophors. They postulated that dynamic quenching indicated the conformational fluctuation of protein molecules. Acrylamide, as a neutral probe, was also introduced for studying the bimolecular rate constant of collision between the probe and the indole side chains, first for that of the RNase T_1, later for several other proteins (Eftink & Ghiron, 1975, 1976b, 1977). These experiments also suggested the nanosecond time scale structural fluctuations of proteins.

According to the results presented in this paper, acrylamide significantly decreased the intrinsic tryptophan fluorescence of phosphorylase *b*. The modified Stern–Volmer plot provided solid evidence that all tryptophans were accessible to the quencher. This meant that the protein was permeable for the acrylamide, since with potassium iodide as ionic quencher about nine tryptophans per monomer were found inaccessible to the iodide, obviously because of their location in the hydrophobic interior of the enzyme molecule. These results are in good agreement with the X-ray crystallographic estimation of Sprang et al. (1979). The collisional fashion of quenching was supported by the nanosecond lifetime data on the tryptophan residues, since the singlet lifetime was shortened by 30% in the presence of 0.4 M acrylamide.

The activation energy of quenching characteristic of the dynamics and structuredness of phosphorylase *b* was 30.9 kJ/mol, i.e., about twice as much as in the case of free tryptophans (Eftink & Ghiron, 1976a). This value may vary from protein to protein according to the different structural characteristics of the structure around the tryptophan residues.

For the purpose of finding a correlation between enzyme function and fluctuation, parallel experiments were carried out which studied the quenching of the tryptophan fluorescence

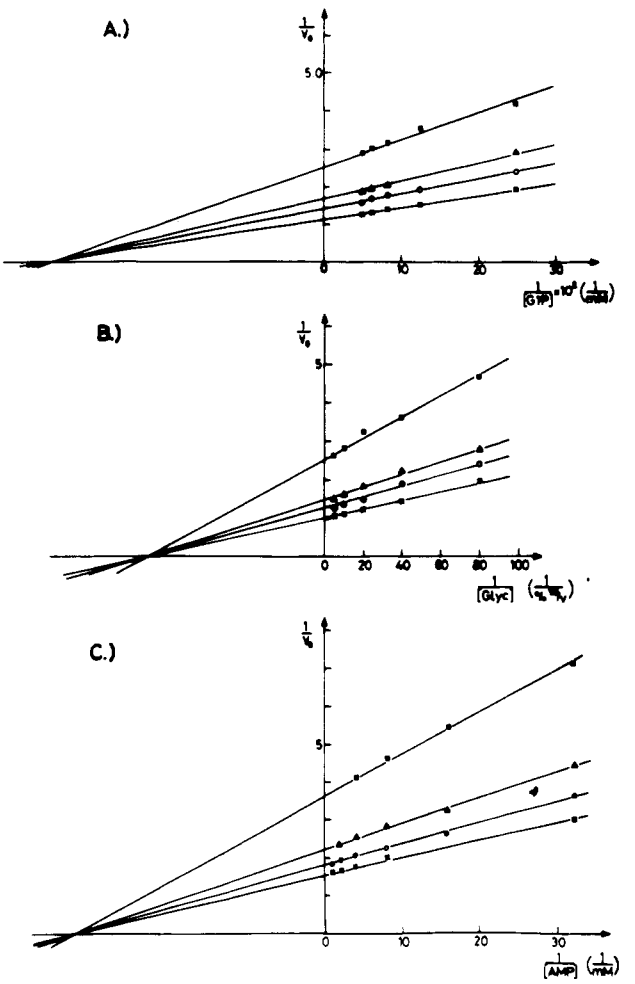

FIGURE 6: Lineweaver–Burk plots of the ligand binding of phosphorylase *b* in the absence and the presence of acrylamide. (A) The reciprocal activity of the enzyme as a function of G-1-P^{-1}. The other ligands (glycogen and AMP) were applied in saturating concentrations (1% and 1 mM, respectively). Concentration of G-1-P varied from 4 to 20 mM. (B) The reciprocal activity of phosphorylase *b* as a function of $[glycogen]^{-1}$. Glycogen concentration varied from 0.0125 to 0.2% (w/v) while concentrations of G-1-P and AMP were held constant (32 and 1 mM, respectively). (C) The reciprocal activity of the enzyme as a function of $[AMP]^{-1}$. AMP concentration varied between 0.03 and 1 mM when the concentration of other ligands (glycogen and G-1-P) were set to saturation level. Acrylamide concentrations were none (×), 0.2 M (○), 0.4 M (▲), and 0.8 M (□). The enzyme concentration was 5.4×10^{-7} M. The activity measurements were carried out at 30 °C as described in the Experimental Section. The initial rate of the reaction (V_0) was expressed in arbitrary (OD) units.

and the activity of phosphorylase *b* at different acrylamide concentrations.

The decrease observed in the enzyme activity (up to 60%) may be due to several factors. Denaturation studies helped to select experimental conditions to avoid denaturation. The decreased ligand saturation as a source of inhibition caused by acrylamide could also be ruled out, since the K_m values for the activator AMP and substrates G-1-P and glycogen were not changed by acrylamide. These findings exclude any similarity to the effects of organic compounds modifying glycogen phosphorylase activity through gross conformational changes and/or influencing the ligand binding (e.g., Dreyfus et al., 1978; Uhing et al., 1979).

The covalent modification of the SH groups by acrylamide (Cavins & Friedman, 1968) can be disregarded since both fluorescence quenching and enzyme inhibition proved to be reversible in the case of phosphorylase *b*. On the other hand,

the reaction of SH groups would disturb the binding of AMP to phosphorylase *b* (Damjanovich & Kleppe, 1966; Sanner & Trôn, 1975).

Though a neutral probe, acrylamide can principally be bound to some parts of the enzyme, resulting in a higher value of the activation enthalpy of the overall reaction. However, the Arrhenius plots and the calculated activation enthalpies did not show any difference between the acrylamide-treated or the control enzyme.

In light of the above facts, we suggest the following interpretation to the experimental findings: acrylamide, penetrating the protein matrix and colliding with the tryptophans masked for the solvent, causes a dynamic quenching. The number of acrylamide molecules per enzyme, assuming an even distribution between solvent and proteins, seems to be enough to "fill up" the interior of the protein molecules so as to put a restraint on the internal motional freedom of the different parts. In this way, the acrylamide molecules inside the enzyme can, with a certain probability, prevent the development of the correct fluctuation pattern, resulting in the activation of the enzyme–substrate complex (Somogyi & Damjanovich, 1971, 1975; Damjanovich & Somogyi, 1973, 1978). Thus, acrylamide would produce such a noncompetitive inhibition of the enzyme that only the preexponential factor of the maximum velocity changes, while the activation enthalpy remains constant.

This type of noncompetitive inhibition may have a more general implication; i.e., in the case of those noncompetitive inhibitions, where the available physical methods fail to show large-scale structural changes and the strong binding of the inhibitor is unlikely, such a mechanism may occur.

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