SHEAR DEFORMATION EFFECTS IN ENZYME CATALYSIS

METAL ION EFFECT IN THE SHEAR INACTIVATION OF UREASE

MATTHEW TIRRELL AND STANLEY MIDDLEMAN, *Departments of Polymer Science and Engineering and Chemical Engineering, University of Massachusetts, Amherst, Massachusetts 01003 U.S.A.*

ABSTRACT The mechanism of the inactivation of the enzyme urease produced by subjecting its dilute solutions to hydrodynamic shear stresses in the range 0.5–2.5 Pa has been determined. By studying the kinetics of urease-catalyzed urea hydrolysis during application of hydrodynamic shear under varying chemical environments, we demonstrate that micromolar quantities of metal ions, in this case adventitious Fe, can accelerate the oxidation of thiol groups on urease and thus inactivate it when the protein is subjected to a shearing stress of order 1.0 Pa. In the absence of metal ion this stress level is ineffectual. It is proposed that this type of synergy between deformation and chemical environment may be crucial in many situations where biological macromolecules are subjected to mechanical stress.

INTRODUCTION

Applying mechanical forces to a protein macromolecule so as to alter its characteristic function(s) offers a unique insight into the biophysical details of protein macromolecular functions. If macroscopically applied mechanical forces alter the function by deforming the structure of the macromolecule, then the macroscopic force/function relationship contains information on the forces stabilizing the protein structure. For this reason, studies of the mechanical instability of sickle cell hemoglobin like that by Asakura and co-workers (1–5) may aid in the understanding of sickle cell disease. Likewise, Charm and Wong (6) suggest physiological implications for the regulation of circulatory protein turnover from their results on reduction of fibrinogen clottability during shear. The ATPase activities of myosin and actomyosin have been shown to increase when glycerinated muscle fibers are subjected to a tensile strain (7) or when solutions are subjected to a shearing deformation (8), in the presence of ATP. When solutions of myosin and actomyosin are sheared in the absence of ATP, catalytic activity decreases (8). We have previously reported (9) and subsequently studied in greater detail (10) the effect of shearing deformation on the solution reaction kinetics of urea hydrolysis catalyzed by the enzyme urease. Under the conditions of the earlier

---

Dr. Tirrell's present address is: Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, Minn. 55455.

study (9) the catalytic activity of urease was found to be reduced during the deformation. Other reports of inactivation of enzymes due to shearing have appeared in recent years (11). In *no* previous case has the mechanism of shear-induced activity alteration been demonstrated, and therefore the potential for new understanding of the mode of operation of biological macromolecules via this technique has remained unrealized. We present evidence here that the mechanism of the previously reported irreversible inactivation of urease (9) is a shear-promoted, metal ion-catalyzed oxidation of an essential sulfhydryl group or groups on urease. The promotion of the inactivation by shearing forces must be due to a shear-induced conformational change in the urease structure.

METHODS

The experimental technique has been described in detail (10). To summarize briefly, the urease-catalyzed hydrolysis of urea was conducted in a coaxial cylinder viscometer designed to provide, as nearly as possible, a homogeneous velocity field throughout the entire reaction volume. In these new experiments the urease used was Sigma crystalline type C-3 (Sigma Chemical Co., St. Louis, Mo.), more than a hundred-fold purer than the urease preparation used in our preliminary work (9,10). Urea concentration was followed by periodic sampling of the reaction mixture and analysis of the sample according to the method of Levine et al. (12). The initial urea concentration in all experiments was 80 mM. The urease concentration in all experiments was 0.01 mg/ml in 0.16 M phosphate-citrate buffer (pH 7.0). The urease was dialyzed against large volumes of this buffer before preparation of the reaction solution to remove any traces of chemicals used in the crystallization. Otherwise, all reagents, additives, and buffer salts were used as received. (See ref. 10 for complete detail on suppliers, lot numbers, etc.) Additives were included in the reaction mixtures as noted. The viscosity of the reaction medium was 0.0013 Pa · s (1.3 cP).

RESULTS AND DISCUSSION

Fig. 1 shows the effect of varying conditions of shear rate on the kinetics of urease-catalyzed urea hydrolysis. At zero shear rate, first-order kinetics are observed. During shearing, however, the rate of hydrolysis decreases with time, the decrease occurring somewhat more rapidly at the higher shear rate. Also shown are data taken after stopping the shearing forces at various times. First-order kinetics are resumed but at a rate less than that from a urease preparation with no shear history. It is apparent, then, that under these deformation conditions in this reaction medium, the inactivation is largely irreversible. This communication offers an explanation for the mechanism of this irreversible inactivation.

Understanding the irreversible portion of the inactivation began with the discovery that addition of ethylenediaminetetraacetate (EDTA) sufficient to make the reaction solution 1 mM eliminates the irreversible inactivation. This means one of two things: either (*a*) EDTA binds directly to urease to protect its structure; or (*b*) metal ions play some role in the shear inactivation mechanism, and chelation by EDTA renders them impotent. It was found that data qualitatively identical to that with 1 mM EDTA

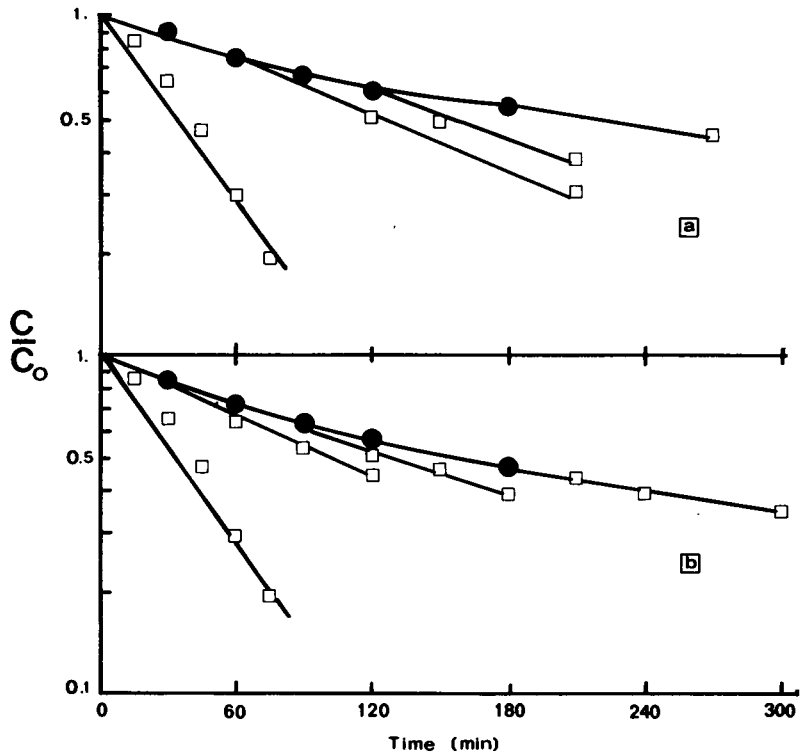

FIGURE 1 Urea concentration vs. time during and after variable periods of steady shear. *a*. □, zero shear rate; ●, 1,717 s^{-1}; *b*. □, zero shear rate; ●, 288 s^{-1}

could be obtained by pretreating (slurrying) all the components of the reaction mixture with a chelating resin Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.), then removing the Chelex before shearing. Chelex 100 consists of one half of the symmetrical EDTA molecule immobilized on polystyrene beads. This demonstrates that the chelon need not be present during shearing to afford protection. Thus, a direct protection is excluded and a role of metal ions indicated. Metal ion concentrations were then checked via atomic absorption spectroscopy and both Fe and Ni were found to be present in the normal reaction medium at levels of 15 and 22 μM, respectively; Cu was not present in significant, detectable quantities. A likely role for these metal ions is that of catalyzing the oxidation of some essential group on the urease molecule, cysteine sulfhydryls being the most likely candidates. Fig. 2 shows the results of addition of varying amounts of the thiol-reducing agent β-mercaptoethanol (β-met) to the deforming reaction medium along with some data of Fig. 1 reproduced for comparison. Even during application of the maximum hydrodynamic shearing force attainable, at 1,717 s^{-1}, in the presence of 1.0 or 0.1 mM β-met, the kinetics are unaltered from the zero shear kinetics. If the β-met concentration is reduced still further to 0.001 mM, inactivation similar in character to that of Fig. 1 is seen. The conclusion

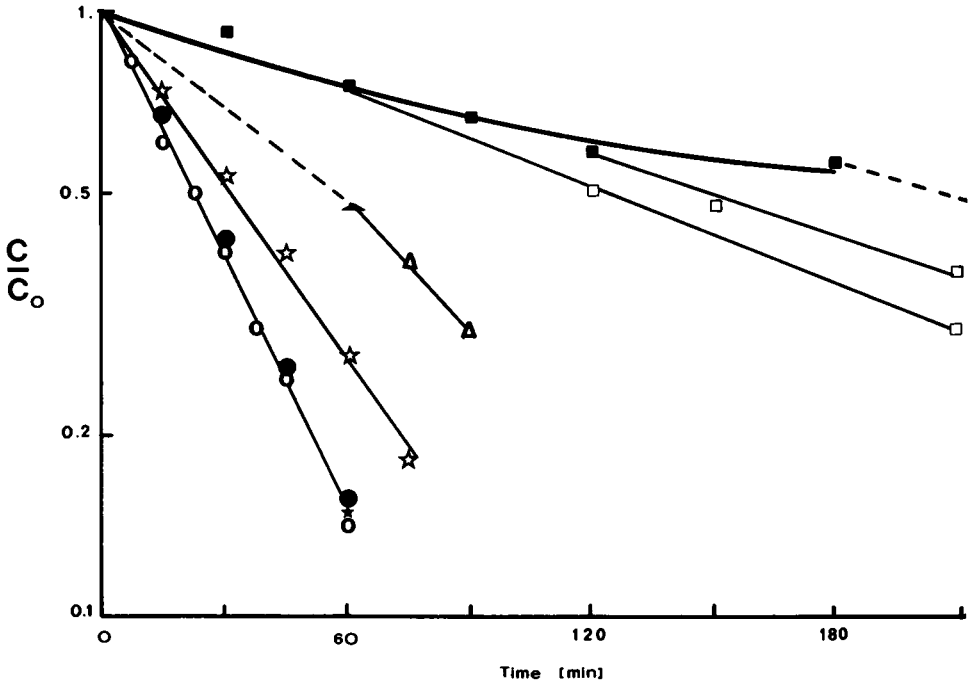

FIGURE 2 Urea concentration vs. time with various levels of β-mercaptoethanol. ☆, zero shear, no additive; ○, zero shear, 1 mM β-met; ●, 1,717 s^{-1}, 1 mM β-met; ★, 1,717 s^{-1}, 0.1 mM β-met; ▲, △ 1,717 s^{-1}, stop shear, 0.001 mM β-met; ■, □ 1,717 s^{-1}, stop shear, no additive (reproduced from Fig. 1 for comparison).

is that keeping sulfhydryl groups in their reduced state will completely prevent shear inactivation of urease at these stress levels (less than 2.5 Pa; 1 Pa = 10 dyn/cm^2), irrespective of what may be happening to the conformation of the macromolecule.

On the evidence presented thus far, this conclusion may be disputed by pointing out that the predominant urease structure (mol wt 480,000 daltons) (13) tends to oligomerize up to at least a pentamer (ie., mol wt 2,400,000 daltons) (13) while β-met completely reverses this process, leaving only the monomer (13), which is presumably less shear-susceptible. To clear up this point, some direct evidence of a shear-promoted reaction with a urease thiol group was sought to separate the thiol-protective effects of β-met from its effects on the urease quaternary structure. This was done by measuring the effect of deformation on urease activity in the presence and absence of the sulfhydryl-specific reagent, parachloromercuribenzoate (PCMB). The reaction medium was Chelex, pretreated so that no metal ion effect was possible (*vide supra*). Fig. 3 shows the results of these experiments. It is seen that in the absence of PCMB (and of metals), shearing at 1717 s^{-1} produces about 13% inactivation, whereas in the presence of 1 μM PCMB, shearing at 1,171 s^{-1} produces about 38% inactivation. Shearing forces of this magnitude are thus seen to be roughly three times as effective in promoting in activation of urease in the presence of 1 μM PCMB as in its absence. Even at

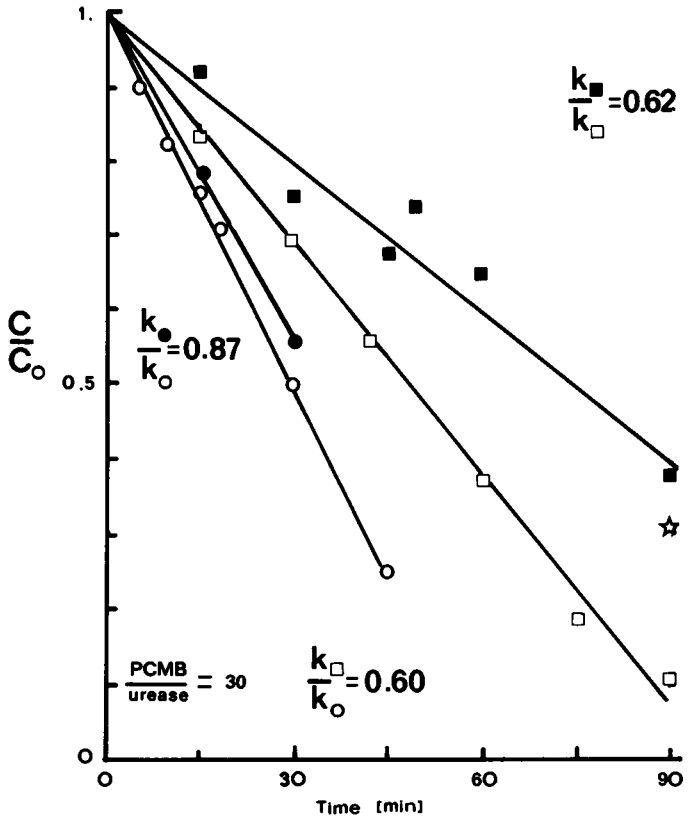

FIGURE 3 Urea concentration vs. time, effect of PCMB. ○, zero shear, no additive; ●, 1,717 s^{-1}, no additive; □, zero shear, 1 μM PCMB; ■, 1,717 s^{-1}, 1 μM PCMB; ☆, 288 s^{-1}, 1 μM PCMB.

288 s^{-1}, PCMB seems to produce markedly enhanced shear inactivation. The evidence is thus decisive that a shear-promoted reaction of a low molecular weight moiety and a urease thiol group or groups takes place.

Finally, some data were obtained with a urease preparation treated with Chelex, to which was subsequently added $Fe(NO_3)_3$ sufficient to make the concentration in the final reaction mixture 5 μM. The kinetic data shown in Fig. 4 obtained under these chemical and deformation conditions are quite similar to those of Fig. 1. No similar inactivation was induced when 5 μM Ni^{2+} was added instead of Fe^{3+}.

From these results, we conclude that the inactivation of urease during shearing at these stress levels, in the presence of micromolar quantities of metal ions, is due to a shear-promoted metal ion-catalyzed oxidation of an essential thiol group(s) on urease.

This and our previous work (9) indicate that the responsible ion is Fe^{3+}, presumably adventitious. There is an interesting parallel between these results and those of Fishbein et al. (14), who reported that inactivation of urease at pH 7–10 by concentrated urea, guanidine, or heat was markedly delayed by EDTA and other chelating agents. They tentatively attribute this to the Ni^{2+}-catalyzed oxidation of a urease thiol, the

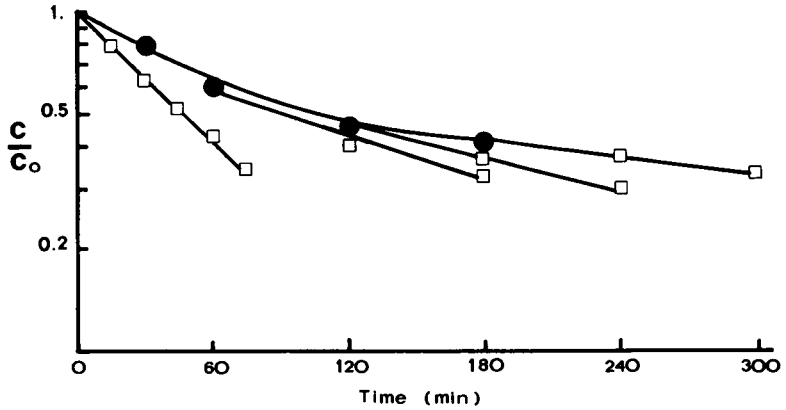

FIGURE 4 Urea concentration vs. time during and after variable periods of steady shear in presence of 5 μM Fe $(NO_3)_3$. □, zero shear; ●, 1,717 s^{-1}.

required nickel atom being released from another urease molecule as the protein unfolds due to the action of the denaturing agent. This in part led to further conclusive evidence that urease is, in fact, the first and only known natural nickel metalloenzyme (14,15). In the unadulterated reaction medium of the present work, the demonstrated abundance and efficacy of adventitious Fe makes it a more likely candidate.

The chemical details of these experiments should not obscure the physical basis of the phenomenon we report here. From a physical viewpoint, the results show that hydrodynamic stresses of the order of magnitude of 2.5 Pa will alter the conformation of urease sufficiently to promote inactivation by a microsolute (e.g. Fe^{3+} or PCMB). In addition, we have shown that this stress level is insufficient to alter irreversibly the activity of urease directly (ie. when the microsolutes are absent or ineffectual). It is apparent then that the forces necessary to render the pertinent sulfhydryl groups accessible to oxidation are different from those necessary to distort the geometry of the active site(s) significantly. More explicit interpretation of the physical processes involved are hampered by two factors: (*a*) the primary and three-dimensional structures of urease are as yet unknown, and (*b*) it is not clear how to estimate properly the deformability of a macromolecular structure stabilized by specific energetic interactions and apolar or hydrophobic forces. For the sake of making some quantitive estimates, consider the following: the urease monomer is nearly spherical with equatorial cross-sectional area of 125 nm^2; under the conditions of our experiments about 35 wt% of the urease exists as linear aggregates up to a pentamer (10), so the maximum molecular cross-section is of order 1,000 nm^2. For any of these species, in a sheared medium the maximum tension experienced by any segment of the backbone chain is of the order of magnitude of the fluid shear stress (viscosity × shear rate), multiplied by the macromolecular cross-section. Thus the maximum tension in any urease species in the deformation conditions of the present work is of order 2.5×10^{-15} N. This same bulk

shear stress has been shown by light scattering to produce about 40% elongation of a randomly coiling polyisobutylene macromolecule (16), but the relevance of this to conformational distortion of proteins is uncertain. If a protein contains any relatively large "random" portions, these may be the most easily distorted by stress, and a mechanism such as this may account for the more facile shear exposure of sulfhydryl groups relative to direct active site distortion. Comparison of the present values of stress with the reported moduli of protein macromolecules (10,17) or with hydrogen bond force constants (10,18) leads to the conclusion that the gross molecular strains under these conditions would be less than 0.01 nm. The strains required to produce the effects we have observed are not known.

We note that there have been reports that hydrodynamic stresses two orders of magnitude lower ($\sim$0.02 Pa) than in the present work produce significant irreversible inactivation of the enzymes catalase, carboxypeptidase, and rennet (19). This is difficult to understand in light of our work, unless metal ions played some undetected role or there is something uniquely mechanically stable about the structure of urease. Subsequent investigations on the effect of mechanical forces on biologically active protein molecules should take account of this type of potential synergy between deformation and chemical environment. In this connection, we also note that Adam and Zimm (20) have shown 10^{-5} molar quantities of La^{3+} to produce a 10-fold increase in the rate of shear degradation of DNA at constant shear stress.

Received for publication 21 December 1977.

REFERENCES

1. AKASURA, T., P. L. AGARWAL, D. A. RELMAN, J. A. MCCRAY, B. CHANCE, E. SCHWARTZ, S. FRIEDMAN, and B. LUBIN. 1973. Mechanical instability of the oxy-form of sickle haemoglobin. *Nature (Lond.).* **244**:437–438.
2. AKASURA, T., T. OHNISHI, S. FRIEDMAN, and E. SCHWARTZ. 1974. Abnormal precipitation of oxyhemoglobin S by mechanical shaking. *Proc. Natl. Acad. Sci. U.S.A.* **71**:1594–1598.
3. AKASURA, T., K. ADACHI, M. SONO, S. FRIEDMAN, and E. SCHWARTZ. 1974. Mechanical instability of hemoglobin subunits: an abnormality in β^S subunits of sickle hemoglobin. *Biochem. Biophys. Res. Commun.* **57**:780–786.
4. ADACHI, K., and T. ASAKURA. 1974. Effect of 2,3-diphosphoglycreate and inositol hexaphosphate on the stability of normal and sickle hemoglobins. *Biochemistry.* **13**:4976–4982.
5. ONISHI, T., T. ASAKURA, R. L. PISANI, K. J. ROSENBAUM, and H. L. PRICE. 1974. Effect of anesthetics on the stability of oxyhemoglobin. *Fed Proc.* **33**:509.
6. CHARM, S. E., and B. L. WONG, 1970. Shear degradation of fibrinogen in the circulation. *Science (Wash. D.C.).* **170**:466–468.
7. OHNISHI, T., and T. OHNISHI. 1963. Adenosine triphosphotase activity of glycerinated muscle fibers in a mechanical field. *Nature (Lond.).* **197**:184–185.
8. VOROB'EV, V. I., and L. V. KUKHAREVA. 1965. Changes in adenosinetriphosphatase activity of myosin during deformation in a hydrodynamic field. *Dokl. Akad. Nauk SSSR (Engl. Transl.). Biochem. Sect.* **165**:435–438.
9. TIRRELL, M., and S. MIDDLEMAN. 1975. Shear modification of enzyme kinetics. *Biotechnol. Bioeng.* **17**: 299–303.
10. TIRRELL, M. 1977. Reaction kinetics and structure of enzyme macromolecules subjected to hydrodynamic shearing forces: enzyme mechanochemistry. Ph.D. dissertation, University of Massachusetts, Amherst, Mass. University Microfilms, Ann Arbor, Mich.

11. TIRRELL, M. 1978. Stress-induced structure and activity changes in biologically active proteins. *J. Bioeng.* In press.
12. LEVINE, J. M., R. LEON, and F. STEIGMANN. 1961. A rapid method for the determination of urea in blood and urine. *Clin. Chem.* **7**:488–493.
13. FISHBEIN, W. N., K. NAGARAJAN, and W. SCURZI. 1973. Urease catalysis and structure. IX. The half-unit and hemipolymers of jack bean urease. *J. Biol. Chem.* **248**:7870–7877.
14. FISHBEIN, W. N., M. J. SMITH, K. NAGARAJAN, and W. SCURZI. 1976. The first natural nickel metalloenzyme: urease. *Fed. Proc.* **35**:1680.
15. DIXON, N. E., C. GAZZOLA, R. L. BLAKELEY, and B. ZERNER. 1975. Jack bean urease. A metalloenzyme. A simple biological role for nickel. *J. Am. Chem. Soc.* **97**:4131–4132.
16. COTTRELL, F. R., E. W. MERRILL, and K. A. SMITH. 1969. Conformation of polyisobutylene in dilute solution subject to a hydrodynamic shear field. *J. Polym. Sci. Part. A-2 Polym. Phys.* **7**:1415–1434.
17. OOSAWA, F., and S. ASAKURA. 1975. *Thermodynamics of the Polymerization of Protein.* Academic Press, Inc., New York. 162–168.
18. MIZUSHIMA, S. 1954. Structure of Molecules and Internal Rotation. Academic Press, Inc., New York. 146–152.
19. CHARM, S. E., and B. L. WONG. 1970. Enzyme inactivation with shearing. *Biotechnol. Bioeng.* **12**: 1103–1109.
20. ADAM, R. E., and B. H. ZIMM. 1976. Shear degradation of DNA. *ACS Polym. Prepr. Am. Chem. Soc. Div. Polym. Chem.* **17(2)**:870–875.