ies allow for a distinct reaction coordinate diagram to be associated with each value of ν_{Fe-His}. The relaxation experiments indicate that the potential surface controlling ligand bonding can change on the time scale of geminate recombination. On the basis of these findings, a model is described incorporating both sequential and parallel processes that can account for species-specific and solution-dependent variations in ligand reactivity within a given quaternary state.

Note added in proof: In recent studies we have measured both the picosecond geminate rebinding of O_2 in a large variety of vertebrate hemoglobins (*49*) and the corresponding time-resolved, low-frequency Raman spectra of the deoxy photoproduct occurring within 25 psec of photodissociation (*50*). These studies reveal that the values of ν_{Fe-His} in the 25-psec transients are the same as in 10-nsec transients discussed here and that the yield for the picosecond geminate rebinding correlates with the ν_{Fe-His} values of the associated deoxy transients. These results confirm the conclusions expressed in this article regarding the correlation between the geminate yield and ν_{Fe-His} that were inferred from the nanosecond studies.

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51. Much of the work described in this article was conducted in collaboration with T. W. Scott, R. Stepnoski, M. Ondrias, D. Rousseau, S. Simon, K. B. Lyons, and V. Macdonald. Their respective contributions are gratefully acknowledged.

RESEARCH ARTICLE

The Mechanism of Irreversible Enzyme Inactivation at 100°C

Tim J. Ahern and Alexander M. Klibanov

As the temperature is increased, all enzymes eventually lose their catalytic activity. This thermal inactivation of enzymes may be either reversible or irreversible, depending on whether return to ambient temperature results in the return of enzymatic activity within a reasonable period of time (*1*). Reversible thermal inactivation (denaturation) of enzymes is caused by temperature-induced conformational transitions in the protein molecule; this phenomenon has been extensively studied and its mechanisms are well understood (*2–5*). The pathways and mechanisms of irreversible thermoinactivation of enzymes, however, remain obscure, primarily because of severe conceptual and experimental problems encountered in their investigation. Knowledge of these mechanisms is essential for the development of rational approaches to enzyme stabilization (*1, 6*), with special application to protein engineering (*7*) and to the enhancement of enzyme thermostability (*8*).

A number of chemical reactions can take place in proteins at high temperature, especially at extremes of *p*H (*9*). But little is known concerning the processes that actually lead to enzyme inactivation in the *p*H range of relevance to enzymatic catalysis (*p*H 4 to 8) and their relative effects. We therefore undertook the following study.

We used hen egg-white lysozyme as a model because it is a small monomeric enzyme containing no nonprotein components and its structure and properties have been thoroughly investigated (*10*).

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Conformational compared to covalent processes in irreversible thermoinactivation. When an aqueous solution of lysozyme at *p*H 6 (optimum of the enzymatic activity) was heated at 100°C, the enzyme immediately and totally lost its activity with respect to lysis of dried *Micrococcus lysodeikticus* cells (*11*). This was not surprising since lysozyme is completely reversibly denatured at that temperature (*12*). If the enzyme solution was promptly cooled to 25°C, its catalytic activity was fully regained. However, prolonged incubation at 100°C resulted in only a fraction of the initial activity being recovered after cooling. This irreversible thermal inactivation of lysozyme follows first-order kinetics at *p*H 4, 6, and 8, and the time course of inactivation is independent of the initial enzyme concentration over several orders of magnitude (Fig. 1). These results suggest that irreversible thermoinactivation of lysozyme in the above ranges of *p*H and concentration (in which all subsequent experiments were carried out) is not due to polymolecular processes such as aggregation, which has been often proposed as the sole mechanism of irreversible thermoinactivation of enzymes (*13, 14*). Our conclusion was confirmed when gel filtration (Sephadex G-100) of lysozyme, irreversibly thermoinactivated (by 95 percent) at *p*H 4, 6, and 8 yielded only one peak coinciding with that for the native enzyme.

It was important to determine whether irreversible thermoinactivation of lysozyme is caused by conformational or covalent processes. In the absence of aggregation, the conformational processes result in incorrectly folded and enzymatically inactive protein molecules that are kinetically or thermodynamically stable at elevated temperatures (*15*). These structures are so "scrambled" that they persist even after cooling because a high kinetic barrier prevents spontaneous refolding to the native conformation. To distinguish between such conformational mechanisms of thermoinactivation and those of a covalent nature, we adopted the following two criteria:

1) Concentrated solutions of strong denaturants (such as guanidine hydrochloride or acetamide) disrupt noncovalent interactions in protein (*3–5*). Such agents, especially at high temperature, should maintain the enzyme molecules in a highly unfolded form and thereby prevent formation of incorrectly (as well as correctly) folded structures. Hence, heating enzymes in the presence of denaturants should stabilize them against irreversible thermoinactivation (*16*) due to conformational processes. Conversely, denaturing agents are not expected to affect the rates of most covalent reactions. The above reasoning constitutes the first criterion: if addition of a denaturant stabilizes an enzyme against irreversible thermal inactivation, then conformational processes are involved; if there is no effect, the rate-limiting step consists exclusively of covalent processes.

2) Reduction of disulfide bonds in enzymes in the presence of high concentrations of denaturants leads to catalytically inactive, random-coiled configurations of the macromolecules (*3, 17*). Since incorrectly folded (scrambled) enzyme structures involve no new types of noncovalent interactions compared to native, the same treatment should convert them to random coils, too. Reoxidation of random coils under native conditions after removal of the denaturant results (at least for proteins consisting of a single polypeptide chain) in the recovery of enzymatic activity (*14, 17, 18*). That opens the way to reactivation of "irreversibly" thermoinactivated enzymes (*15*) and constitutes the second criterion: if reduction and unfolding of irreversibly thermoinactivated enzymes, followed by reoxidation and refolding results in at least partial recovery of the enzymatic activity lost due to heating, then such inactivation involves formation of incorrect structures; the lack of reactivation would suggest that only covalent processes are operating. The above criteria provided a basis for the determination of the exact mechanisms of irreversible thermoinactivation of lysozyme at 100°C at *p*H 4, 6, and 8.

Abstract. *The mechanism of irreversible thermoinactivation of an enzyme has been quantitatively elucidated in the* p*H range relevant to enzymatic catalysis. The processes causing irreversible inactivation of hen egg-white lysozyme at 100°C are deamidation of asparagine residues, hydrolysis of peptide bonds at aspartic acid residues, destruction of disulfide bonds, and formation of incorrect (scrambled) structures; their relative contributions depend on the* p*H.*

Thermoinactivation at p*H 4.* The roles of conformational and covalent mechanisms were compared. Neither 6*M* guanidine hydrochloride nor 8*M* acetamide

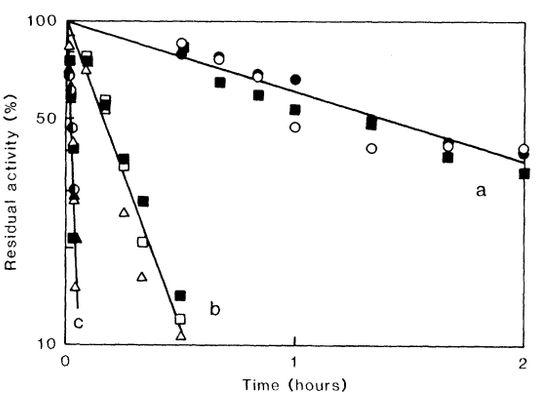

Fig. 1. The time course of irreversible inactivation of lysozyme at 100°C as a function of *p*H and the enzyme concentration. (Curve a) *p*H 4 (0.1*M* sodium acetate), (curve b) *p*H 6 (0.01*M* sodium cacodylate), and (curve c) *p*H 8 (0.1*M* sodium phosphate). The concentrations (μ*M*) of lysozyme were ○, 1000; ●, 100; □, 50; ■, 10; △, 5; ▲, 1; ◐, 0.5. Stoppered test tubes containing solutions of lysozyme [Sigma, grade I, further purified to homogeneity by ion-exchange chromatography (*44*)] were placed in a 100°C glycerol bath. Periodically, portions were withdrawn and assayed for the lysozyme activity toward *M. lysodeikticus* cells (*11*) at *p*H 6.

Table 1. Rate constants of irreversible inactivation of lysozyme at 100°C.

Irreversible thermoinactivation	Rate constant ($hour^{-1}$)		
	*p*H 4	*p*H 6	*p*H 8
Directly measured overall process*	0.49	4.1	50
Due to individual mechanisms			
Deamidation of Asn residues†	0.45	4.1	18
Hydrolysis of Asp-X peptide bonds‡	0.12	0	0
Destruction of cystine residues§	0	0	6
Formation of incorrect structures‖	0	0	32

*Determined from Fig. 1. †Determined by nonequilibrium polyacrylamide gel electrophoresis. ‡Determined by SDS gel electrophoresis of the reduced enzyme; it is assumed that hydrolysis of a single peptide bond results in enzyme inactivation. §Determined by titration of SH groups formed upon reduction of cystine residues; it is assumed that destruction of any cystine residue results in enzyme inactivation. ‖Determined as the difference between the time courses of irreversible thermoinactivation of lysozyme in the absence and in the presence of 8*M* acetamide.

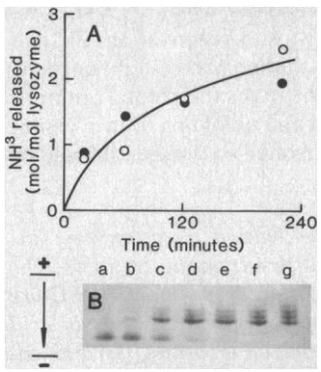

Fig. 2. The time course of deamidation of lysozyme at 100°C and *p*H 4 (0.1*M* sodium acetate) determined by (A) ammonia evolution and (B) change in isoelectrophoretic mobility. (A) The amount of NH_3 formed was either measured directly (*45*) (○) or calculated on the basis of the data presented in (B) (●). Sealed ampules containing 50 μ*M* lysozyme were incubated for the indicated periods of time at 100°C, cooled, and assayed for NH_3 (*45*). (B) Solutions of lysozyme (200 μ*M*) thermoinactivated for (a) 0, (b) 5, (c) 20, (d) 60, (e) 80, (f) 120, and (g) 220 minutes were subjected to nonequilibrium polyacrylamide gel electrophoresis. The samples of the enzyme were applied on 4.0 percent polyacrylamide gel (0.24 percent bis) containing 5.0 percent of *p*H 9 to 11 LKB ampholyte that had been prefocused at 200 volts for 1 hour; then they were focused for 2 hours at 200 volts. Protein bands were stained with Coomassie blue R-250 and quantified by means of an integrating gel scanner (*39*).

appreciably affected the time course of irreversible thermoinactivation of lysozyme at 100°C. In addition, the reduction-reoxidation procedure (*19*) which, when applied to the native enzyme regenerates about two-thirds of the initial activity, failed to recover any enzymatic activity lost during heating. Hence, formation of incorrect structures plays no role in irreversible thermoinactivation of lysozyme at *p*H 4.

The integrity of the polypeptide chain of heated lysozyme was then examined. The time course of the disintegration of lysozyme's polypeptide chain follows first-order kinetics (*20*), yielding a rate constant of 0.12 $hour^{-1}$. The only new COOH-terminal amino acid formed was aspartic acid, as determined by hydrazinolysis (*21*) of thermoinactivated and carboxymethylated lysozyme. This result is in agreement with the data (*22*) on hydrolysis of proteins in dilute acid solutions which indicate that the Asp-X bond (where X is the amino acid residue bonded to the α-carboxyl group of Asp) is the most labile peptide bond under those conditions. Analysis of the NH_2-terminal (*23*) revealed that at least four of the seven peptide bonds adjacent to the α-carboxyl groups of Asp residues in lysozyme are labile, although the Asp-Tyr bond may be somewhat more sensitive than others.

Comparison of the rate constant of the polypeptide chain disintegration due to hydrolysis of Asp-X bonds with that of irreversible thermoinactivation of lysozyme at *p*H 4 (Table 1) shows that, even if each cleavage results in enzyme inactivation, hydrolysis alone cannot be the sole mechanism. Therefore, decomposition of individual amino acids was next examined. No appreciable changes, with the possible exception of the deamidation of asparagine and glutamine residues, were detected (*24*).

We then determined whether such deamidation is in fact another and perhaps the major process causing irreversible thermoinactivation of lysozyme. The time course of the initial evolution of NH_3 during heating of a lysozyme solution at 100°C and *p*H 4 (Fig. 2A) indicates that deamidation occurs at a significant rate. Studies of model peptides have demonstrated that Asn and Gln residues release ammonia as a result of deamidation even at slightly elevated temperatures and that the amide bond in Asn residues is more labile than that in Gln (*25*). In particular, studies of model pentapeptides incorporating hen egg-white lysozyme sequences (*26*) revealed that the most labile of the three Gln residues in the enzyme is nearly one-and-a-half orders of magnitude more stable than the most stable of the 14 Asn residues. We conclude that the initial release of ammonia from lysozyme at 100°C is overwhelmingly due to deamidation of Asn residues.

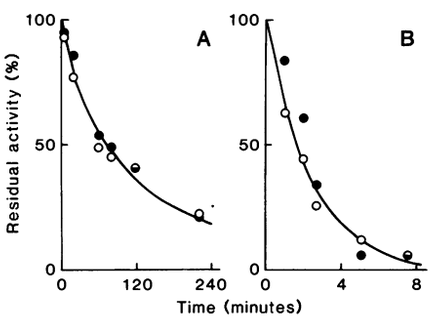

Fig. 3. Comparison of experimentally observed (●) and calculated (○) time courses of irreversible inactivation of lysozyme at 100°C. (A) *p*H 4, (B) *p*H 8. Thermoinactivation experiments were carried out as described in the legend to Fig. 1 except that, at *p*H 8, enzyme solutions contained 8*M* acetamide to eliminate the conformational component to the inactivation process. The residual lysozyme activity was calculated as $[1 - (a_i + a_j - a_i \cdot a_j)] \cdot 100$ percent, where a_i and a_j are fractions of the activity lost due to two independent covalent processes resulting in thermoinactivation. At *p*H 4, *i* is deamidation of Asn residues and *j* is hydrolysis of Asp-X peptide bonds; at *p*H 8, *i* is deamidation of Asn residues and *j* is destruction of cystine residues.

This process was also investigated by isoelectrofocusing. Samples of lysozyme thermoinactivated for different periods of time were subjected to nonequilibrium polyacrylamide gel electrophoresis (Fig. 2B). As a result of heating, the native band (the first from the bottom) gradually disappears while new, distinct bands of more electronegative species appear. It is reasonable to assume that each of these new bands consists of protein that has undergone differing degrees of deamidation: the second from the bottom corresponds to monodeamidated lysozyme, the third to dideamidated, and so forth. This assumption afforded direct determination (*27*) of the rate of NH_3 evolution on the basis of the data in Fig. 2B and resulted in the time course shown by closed circles in Fig. 2A, which is in satisfactory agreement with the NH_3 release data and thus affirms the mutual cause of the two.

Electrostatic attraction between the positively charged lysozyme molecules and negatively charged *Micrococcus lysodeikticus* cells is thought to play an important role in their interaction (*28*). In addition, three Asn residues in lysozyme are a part of its active center and form hydrogen bonds with the substrate (*10*). Hence it appeared convincing that deamidation of Asn residues (which decreases the net positive charge of the protein) would result in inactivation of lysozyme. A quantitative relation between deamidation and inactivation was demonstrated by isoelectric focusing of the thermoinactivated lysozyme, and isolating the variously charged species. The relative distribution of the deamidated species (Fig. 2B) and their relative specific activities (*29*) were used to calculate the rate constant of inactivation of lysozyme due to deamidation of Asn residues to be 0.45 $hour^{-1}$. The calculated and experimental data (Fig. 3A) are in excellent agreement, indicating that those processes are the cause of irreversible thermoinactivation of lysozyme at *p*H 4 and 100°C.

Thermoinactivation at p*H 6*. The time course of irreversible thermoinactivation of lysozyme at 100°C and *p*H 6 is described by the rate constant of 4.1 $hour^{-1}$ (Fig. 1, curve b). To reveal the mechanism of this process, we followed the same steps as outlined above for thermoinactivation at *p*H 4 and found that (i) thermoinactivation is not due to formation of incorrect structures; (ii) no cleavage of the polypeptide chain occurs during the time required to thermoinactivate the enzyme by 95 percent; (iii) no destruction of amino acid residues occurs in lysozyme during thermoinactivation (except for deamidation of Asn resi-

dues); and (iv) significant evolution of ammonia (presumably due to deamidation of Asn residues) occurs during thermoinactivation.

Following the same isoelectric focusing strategy as that used at *p*H 4 (Fig. 2B), we calculated the rate constant of thermoinactivation of lysozyme due to heat-induced deamidation to be 4.1 hour^{-1}. The fact that it exactly coincided with that of overall inactivation (Fig. 1) indicates that the cause of irreversible inactivation of lysozyme at *p*H 6 and 100°C is deamidation of Asn residues.

Thermoinactivation at p*H 8*. We found that 8*M* acetamide or 6*M* guanidine hydrochloride decreases the rate constant of irreversible thermoinactivation by a factor of 2.5. This significant stabilization of the enzyme by denaturants indicates that the inactivation is partly due to a conformational process, that is, formation of incorrect structures (*1*). To verify that, we attempted to reactivate the irreversibly inactivated lysozyme (Fig. 1, curve c). Heating a solution of the enzyme at *p*H 8 for 2 minutes resulted in a residual activity of about 20 percent. The sample was then reduced (and unfolded) and reoxidized (and refolded) (*19*). After the adjustment for the regeneration efficiency, more than half of the enzymatic activity lost as a result of conformational processes was returned, thereby confirming their role in thermoinactivation.

The fact that lysozyme thermoinactivates, albeit more slowly, even in the presence of 8*M* acetamide, means that covalent processes are also involved. To elucidate them, lysozyme inactivated at *p*H 8 was examined as described above for *p*H 4 and 6. No cleavages in the polypeptide chain of the thermoinactivated, reduced (*30*), and carboxymethylated (*31*) lysozyme were detected by SDS-gel electrophoresis. Amino acid analysis of the same sample showed only one appreciable change in comparison with the native enzyme—a decrease in the number of cystines (measured as carboxymethylated cysteines). We examined the kinetics of this reaction by reduction of the thermoinactivated enzyme (*30*), removal of the reductant, and titration of the sulfhydryl groups formed according to the modified Ellman's procedure (*32*). Figure 4A shows the time course of the destruction of the first (of four total) cystine per lysozyme at 100°C. The complete time course of destruction of cystines in lysozyme (Fig. 4B) exceeds the time period needed to inactivate the enzyme, and it resembles that of a first-order process, presumably β-elimination (*33*), an indication that all four cystines are comparably labile to heat.

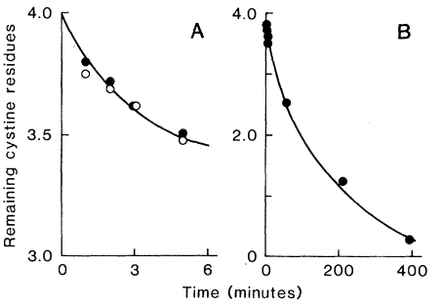

Fig. 4. The time course of destruction of cystine residues in lysozyme upon heating at 100°C and *p*H 8. The amount of remaining cystine residues was based on the number of sulfhydryl groups, detected by spectrophotometric titration with 5,5′-dithiobis(2-nitrobenzoic acid) (*32*), formed after the reduction with dithiothreitol (*30*) and desalting. Lysozyme solutions were heated as described in the legend to Fig. 1 (*p*H 8). Incubation was carried out either in the presence of 8*M* acetamide (○) to eliminate conformational inactivation mechanisms or in the absence of the denaturant (●).

The combined contributions of the conformational process and destruction of S-S bonds do not fully explain the rate of irreversible thermoinactivation of lysozyme at *p*H 8 (Table 1). Since the rate of deamidation of Asn residues increases with *p*H above 6 (*25*), that process was the natural candidate for the missing mechanism. The thermoinactivated lysozyme was subjected to isoelectrofocusing and analyzed as described for *p*H 4. The rate constant of 18 hour^{-1} was obtained.

In order to quantitatively assess the contributions of the individual processes to the overall rate of irreversible thermoinactivation, it was necessary to validate our assumption that the presence of the denaturant does not affect the covalent inactivation reactions uncovered. The time course of the decrease in cystine residues is virtually the same in the presence and in the absence of the denaturant (Fig. 4A). Likewise, 8*M* acetamide had no appreciable effect on the rate constant of heat-induced deamidation of lysozyme at *p*H 8 (determined as in Fig. 2B). These findings are in agreement with out first criterion for distinguishing between conformational and covalent mechanisms in enzyme thermoinactivation.

Figure 3B shows the experimentally observed time course of irreversible thermoinactivation of lysozyme at *p*H 8, compared to the net loss of activity due to destruction of cystine residues and deamidation. The good agreement between the sets of data indicates that these two processes plus the formation of incorrect structures are the cause of irreversible inactivation of lysozyme at *p*H 8 and 100°C.

Because of their general nature, the reactions shown in Table 1 would take place in all proteins at high temperature and thus demarcate the upper limit of thermal stability of proteins—a half-life of no more than a few hours at 100°C in water in the above *p*H range. Furthermore, since water directly or indirectly participates in all the processes causing enzyme thermoinactivation, our results explain why enzymes are extremely thermostable in anhydrous organic media (*34*) or in the dry state (*35*).

Our findings afford rational strategies for the thermostabilization of enzymes by protein engineering (*7, 8*). For instance, replacement of Asn (for example, with Gln, Ile, or Thr) should reduce the rate of deamidation (*25*) and hence should reduce the rate of irreversible thermoinactivation by stabilizing those enzymes that lose their activity due to that process (like lysozyme at *p*H 6). Substituting Glu for Asp residues conserves net charge, yet should enhance thermostability of enzymes that inactivate as a result of hydrolysis of Asp-X peptide bonds [in that the latter are much more labile than Glu-X (*22*)]. The above approaches are different from and yet complementary to those (*36, 37*) derived from comparative studies of proteins from mesophilic and thermophilic organisms where reversible thermodenaturation has been the focal point.

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20. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (*38*) of the reduced (*30*) and carboxymethylated (*31*) thermoinactivated lyso-

zyme revealed that in the course of inactivation the band corresponding to the native enzyme disappeared while several new bands of lower molecular weight appeared. These data indicate that the polypeptide chain of lysozyme is cleaved during thermoinactivation, which was quantitatively assessed by measurement, with the use of gel scanning optical densitometry (*40*), of the intensity of the native band as a function of the time of thermoinactivation.
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23. Lysozyme was reduced, carboxymethylated, dansylated, acid hydrolyzed, and subjected to two-dimensional thin-layer chromatography (*40*). The chromatogram of the 95 percent thermoinactivated enzyme hydrolyzate showed a clear spot corresponding to tyrosine (indicating hydrolysis of the Asp^{52}-Tyr^{53} bond during thermoinactivation) and two very faint spots corresponding to glycine and valine. If heating is continued beyond 95 percent inactivation, then the last two become brighter and a new one corresponding to Ile appears; not only Tyr, but also Gly, Val, and Ile form Asp-X bonds in lysozyme (*10*).
24. Thermoinactivated lysozyme, reduced (*30*) and carboxymethylated (*31*), was completely hydrolyzed in concentrated HCl (*41*) and analyzed by high-performance liquid chromatography with the use of precolumn derivatization with phthaldialdehyde (*42*). None of the amino acid residues detectable by this method were destroyed during thermoinactivation. Acid hydrolysis breaks down tryptophan (*41*), which was, therefore, analyzed (*43*) in the inactivated lysozyme prior to acid hydrolysis; no differences in comparison with the native enzyme were observed. Also, since acid hydrolysis deamidates Asn and Gln (*41*), our method did not distinguish them from Asp and Glu, respectively.
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27. The distribution of the species was measured by gel-scanning optical densitometry and then each of them was assigned a certain amount of NH_3 molecules released (for example, 1 to monodeamidated, 2 to dideamidated, and so forth).
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46. Supported by NSF grant PCM-8316020 (A.M.K.) and a postdoctoral fellowship from Shell Ltd. (T.J.A.). We thank Stephen Zale for helpful discussions and Paul Tesser for conducting preliminary experiments.

21 September 1984; accepted 1 January 1985

RESEARCH ARTICLE

Biochemical Modeling of an Autonomously Oscillatory Circadian Clock in *Euglena*

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There have been many efforts to discover the molecular mechanism (or mechanisms) for the circadian clocks underlying numerous physiological processes (*1*). Hypotheses have been proposed that stress the central role of transcription, protein synthesis and translation on 80*S* ribosomes, adenosine 3′,5′-monophosphate (cyclic AMP), and cellular membranes (*2–4*). There are difficulties inherent in distinguishing between the so-called "hands" of the clock (mechanism-irrelevant events) and the "gears" themselves (clock-relevant processes) which have impeded efforts to test these hypotheses. Phase-shift experiments have been used to ascertain whether or not a given process is an integral part of a circadian clock on the rationale that a transitory perturbation of either the state variables or the parameters that may be used to characterize an oscillation can cause a permanent phase shift in an overt rhythm (*5*). Unfortunately, the converse is not necessarily true; an observed phase shift may have occurred as a result of the effect of the drug or other perturbing agent on some site only secondarily affected by the drug rather than on its postulated primary target (*6*).

The rationale. A circadian oscillator can be expressed mathematically as a set of differential equations comprising both state variables and parameters. The state variables characterize the state of the oscillation, with each set of values defining each phase of the oscillation. The parameters are constants constraining the manner in which the state variables change and determining the dynamics of the oscillations; a different set of parameter values gives a different solution of the rate equations. Any transitory alteration or perturbation of either the state variables or of the parameters can cause a permanent phase shift in an overt rhythm but has no permanent effect on its period; in contrast, permanent changes in the parameter values can alter the steady-state period of the oscillation [see (*5*)].

We have now operationally designated any element as a "gear" (G) of a circadian clock if it can be expressed as a state variable or a parameter; if not, it is a "nongear" (~G). Together, the set of gears would constitute a closed control loop, or oscillator. Because an unperturbed ~G in its normal or physiological oscillatory range would not be expected to regulate the operation of the G's themselves, its artificial perturbation within this range (*7*) should not perturb circadian timekeeping (no steady-state phase shift in an overt rhythm should be observed). Consequently, if an experimental alteration in the level of a target within its normal range perturbs the clock and generates steady-state phase shifts, then that target is most likely a G (criterion A). It is conceivable, however, that the activation and resulting increase in the level of a ~G might perturb timekeeping, whereas its inhibition would not (or vice versa). For example, although the inhibition of protein synthesis can perturb the clock in several organisms (*8, 9*), its activation in both *Neurospora*

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