On Low-Barrier Hydrogen Bonds and Enzyme Catalysis

Although we agree that hydrogen bonds are important in enzyme catalysis, we disagree with the contention of Cleland and Kreevoy (*1*) and Frey *et al.* (*2*) that "low-barrier hydrogen bonds" (LBHBs) can explain enzyme catalysis by supplying up to 20 kcal/mol of energy by "resonance-stabilization." The stabilization of hydrogen bonds (HBs) and hence their catalytic effect is largely electrostatic in condensed phases, including proteins. This is in addition to other "generic" electrostatic effects which are also important. We agree that HBs can contribute up to 5 kcal/mol to transition state stabilization (*3–7*); however, the source of that effect is the reduction in the reorganization energy of the environment (frequently represented by nearby HBs), rather than LBHB stabilization (*5–7*). Of course, the physics of HBs involves more than simple electrostatics. However, as we show below, analyzing the corresponding energetics leads to the conclusion that LBHBs destabilize ionic transition states relative to asymmetric HBs as well as the corresponding case in water and thus leads to "*anti*catalysis."

Enzyme catalysis occurs when the enzyme binds the transition state more strongly than the corresponding transition state in solution. The simplest way an enzyme could accomplish this would be to have strong noncovalent interaction with the transition state. The two main components of such noncovalent interactions are steric (van der Waals) and electrostatic (*8*). As argued by Warshel and Levitt (*9*), the electrostatic effect is the more likely candidate for transition state stabilization, although there may be cases where steric effects are important. Calculations by ourselves and others on trypsin (*10, 11*), subtilisin (*5, 6*), carbonic anhydrase (*12*), triose phosphate isomerase (*13, 14*), and staphyloccocal nuclease (*15*) show that stabilization of the transition state by electrostatic factors is sufficient to account for the major part of the catalytic effects of these systems; no other effects need to be added "deux ex machina." Let us explain why the LBHB is not the "missing link" in enzyme catalysis.

1) The fact that LBHBs can have energies of up to 30 kcal/mol in the gas phase is not relevant to any energy gain these bonds might provide in solution or in the enzyme active site (*16*). In order for the LBHB concept to have an existence separate from electrostatic effects (which include "normal" hydrogen bonds), it should supply the energy by "covalent" interactions. A description of hydrogen bonding that takes polar solvation into account would indicate that the solvation energy of a "polarized" double-well HB is more than enough to offset the "covalent" stabilization. Any transition state stabilization attributed to LBHB can probably be interpreted simply as electrostatic (*17*).

2) The observation of a short hydrogen bond in a polar environment cannot be used to estimate its strength, identify the source of its stabilization, and conclude that it can be used for catalyzing a reaction. A recent study (*18*) showed that a short HB provides only moderate stabilization of an enzyme-inhibitor complex. It is conceivable for HBs to attain high resonance stabilization in the gas phase or in crystals where they actively participate in the collective phenomenon of crystallization by forming infinite chains of HBs strengthened by resonance. However, in a polar liquid or at the enzyme active site, the hydrogen bond will be polarized in such a way as to attain a large solvation energy, and this makes the LBHB unfavorable (Fig. 1). That is, the solvation energy is much larger for the concentrated charge of the X^- H-X configuration than for the delocalized charge of the $X^{-1/2}$ H $X^{-1/2}$ system as is well established by studies of S_N2 reactions and related problems (*19, 20*). Thus a polar solvent (not only water) or a nearby ion will favor the asymmetric non-LBHB configuration. This point is confirmed by calculations by Kidric *et al.* (*21*) and by a more quantitative ab initio free energy perturbation study (Fig. 1). With this fact in mind and with the realization that enzyme active sites stabilize ionic transition states by solvating rather than disolvating them (*16*), we conclude that such sites will stabilize the asymmetric configuration in which the proton would be localized in one of the double wells.

3) The assertion that similar values for the pK_a (negative logarithm of the acidity constant) donor and acceptor are central for stabilizing transition states in enzyme catalysis is not a general rule. For example, as argued by Frey *et al.*, the oxyanion hole of the serine proteases achieves considerable transition state stabilization without pK_a equivalence. This stabilization can be quantitatively modeled by simple electrostatic effects (*5, 6*) without adding any LBHB contributions (which are probably smaller in the enzyme than in the reference reaction in solution). The other cases described by Cleland and Kreevoy (*1*) can be rationalized in a similar way.

4) According to Cleland and Kreevoy (*1*), one of the requirements for forming LBHB is the absence of water molecules. This requirement is difficult to fulfill, as water molecules must be thermodynamically quite stable in the polar environment of the active site (*22*), and would be nearby (and contribute to the polarity of the site) even when they are pushed out of the cav-

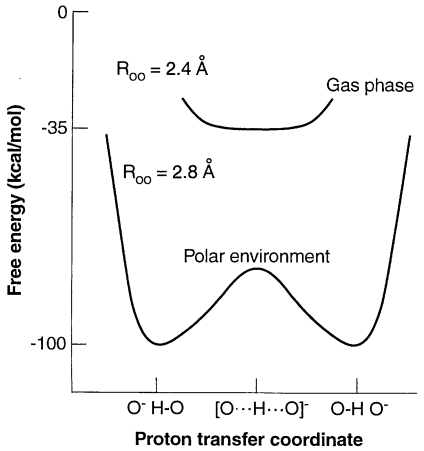

Fig. 1. The energetics of the HO^- HOH system in the gas phase and in polar environment (that is, water). The free energy curves were obtained by free energy perturbation calculations with the use of a combined ab initio molecular mechanics method (*28*). The calculations involve a collinear O^- H-O arrangement. Each free energy curve was obtained with the O-O distance that results in the lowest energy in a given environment. The proton transfer coordinate is taken as the O-H distance. Because a concentrated charge is solvated more strongly than a "distributed" one, the asymmetric configuration is stabilized relative to the LBHB configuration.

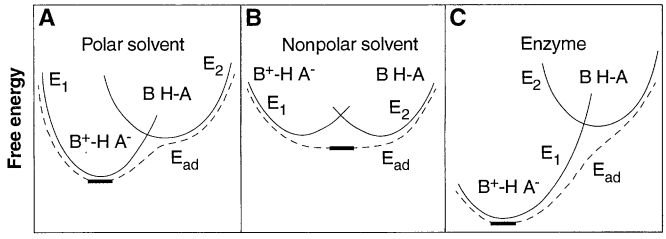

Fig. 2. A schematic representation of the free energy surface perpendicular to the actual reaction coordinate at the transition state of the hydrolysis reaction catalyzed by serine protease. The proton transfer (PT) coordinate corresponds to the position of the proton between Asp^- and His^+ of the "catalytic triad." The lowest point on each adiabatic free energy curve (E_{ad}) corresponds to the transition state of the given system. Note that the relevant reaction is not along the PT coordinate. (**A**) Polar solvent. (**B**) Nonpolar solvent. (**C**) Enzyme.

ity. Many of these water molecules may not show up in the crystallographic structures because of their mobility. An example of the role of active site water molecules in stabilizing transition states is given by Hwang and Warshel [figure 13 in (5)]. Perhaps more importantly, as a functional enzyme must solvate ionic intermediates strongly, it utilizes its polar residues to provide large solvation effects even in a case where the active site is truly isolated from water. Such a polar active site will have a similar effect on LBHB as does water.

To address the LBHB concept in a more quantitative way, we can relate it to the energetics of HBs using the valence bond (VB) representation (*23*). In particular, one can take the Empirical Valence Bond (EVB) Method [for example, (20)], calibrate it by using ab initio gas phase calculations and experimental information about the system in solution, and then use it to analyze LBHB in proteins. Such analysis (*10, 25*) demonstrated the *anti*catalytic nature of LBHBs. The same conclusion would be reached by any treatment that includes the solvation effects in a logical framework. A typical HB system is considered (Fig. 2) in terms of two resonance structures (A^-H-B^+) and (A-H B) with free energy surfaces E_1 and E_2, respectively, and obtain the adiabatic free energy surface (E_{ad}) by mixing these two diabatic states. The degree of mixing of the two states determines the importance of the LBHB effect. When the two states have similar energy, the mixing is large (Fig. 2B) and the corresponding resonance stabilization effect can be identified with the nonelectrostatic LBHB effect. On the other hand, when the energy difference is large, we will have small mixing and therefore a small LBHB effect. All H bonds have some resonance stabilization (or "charge transfer" character), but only if the effect is much larger in the enzyme than in the same system in solution does one need to consider LBHB effect on catalysis. The easiest system with which to explore this issue is the serine protease class studied by Frey *et al.* In contrast to their hypothesis, the ΔpK_a between the catalytic His and Asp is very different from zero. This has been established quantitatively by calculations that considered the covalent interactions (necessary for a "nonelectrostatic" LBHB effect) explicitly (*10, 25*) and reproduced the known facts about the values of pK_a of the relevant groups in the enzyme active site. In this case, the only way to stabilize the system (to lower the minimum of E_{ad}) is to stabilize the ionic state (A^-HB^+), as the enzyme does not interact strongly with the neutral configuration. Trying to exploit the LBHB effect by destabilizing the (A^-HB^+) state and thus driving the ΔpK_a to zero will destabilize the system (Fig. 2B). The enzyme indeed stabilizes the ionic state (*25*). This point is confirmed by the fact that the protein reduces the pK_a of Asp102 by providing a network of HBs to this acid (*10, 24*).

Cleland and Kreevoy (*1*) note in their introduction that "enzymologists have long wondered, for example, how enzymes find it so easy to remove protons from carbons next to carboxylate groups." A similar puzzle is provided by triose phosphate isomerase (TIM), where the enzyme removes H^+ from C near a phosphate dianion. As suggested by one of us (P.A.K.), (*13, 14*) the enzyme can achieve this by electrostatic stabilization of the enediolate mainly by a histidine and a lysine. It has preoriented these groups during synthesis of the enzyme, analogously to how host-guest systems are preorganized for molecule binding. Thus, both the His and Lys^+ play an important role of stabilizing the anionic enediolate transition state and that effect is the key to the catalysis of the enzyme.

In the other enzymes analyzed by Cleland and Kreevoy (*1*) there is no reason to expect that anything other than electrostatics are required to explain the origin of the stabilization of the transition state. Excellent examples are metalloenzymes, such as staphylococcal nuclease (*12, 15*) and thermolysin. In this case a large catalytic contribution is provided by the positively charged metal ion (in its specific protein dielectric). This changes the pK_a of the catalytic water by more than seven units (or 10 kcal/mol) and provides a crucial help in a proton transfer to a base (or to OH^- from the surrounding solvent). The new pK_a might be similar to that of the base, but this does not lead to LBHB stabilization. The enzyme is designed to stabilize the OH^- by its polar active site (particularly the metal ion) and "spreading" this charge between the donor and acceptor by having a free-floating H would cost too much in terms of the solvation of the system.

The problems with the LBHB concept for O^-H-X $\rightleftharpoons$ O-H X^-–type systems can also be illustrated with the oxyanion hole in serine protease. In this and other related cases there are at least two protein HBs and several water molecules stabilizing the oxyanion (*5*). Transferring the proton to oxyanion from one of the HB donors will be opposed by the second protein HB which is aligned to stabilize the oxyanion. This argument is more general: if the enzyme is preorganized to electrostatically stabilize one ionic state of an acid-base couple, it is highly unlikely that anything would be gained by transferring the proton, because that would remove the electrostatic stabilization (*26*). This enzyme preorganization is analogous to that incorporated in simple host-guest systems, in which hosts optimally bind their guests. For example, the binding of adenosine triphosphate by protonated amine analogs (*27*) of crown ethers will, if anything, increase the ΔpK_a of the phosphates and amines.

One may argue that the enzyme microenvironment can in principle do anything, including LBHB formation. There are, however, simple energy constraints that drastically restrict the available options. In particular, if enzyme active sites were really constructed to have a single HB in a nonpolar environment, the transition state would have much less stabilization than in water and resemble the upper part of Fig. 1.

Several of the LBHB examples presented by Cleland and Kreevoy (*1*) involve true proton transfer (PT) steps rather than stabilization of charged transition state. Of course, the transition states of PT steps involve resonance stabilization, as $E_1 = E_2$ at these points. However, the same effect occurs in a nonenzymatic PT reaction and it has no particular catalytic advantage.

Finally, the reader might wonder about the relationship between experiments and the computational work that we cite here. We can only state that we are not aware of any direct measurement of the energetics of LBHB in transition states. On the other hand, calculations that reproduce the observed catalytic effect of HBs [for example, (*25*)] indicate that the corresponding LBHB is *anti*catalytic.

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Response: Quantum mechanics contains no forces other than coulombic, so all of molecular science in some sense "can probably be interpreted as simply electrostatic." It is not useful to do so. Most chemists prefer to reserve the term "electrostatic" for first-order coulombic interactions; that is, for those interactions all aspects of which can be understood by looking at the coulombic interaction of the groups involved, without changing the wave functions determined for them in isolation. By this criterion strong hydrogen bonds are not simply electrostatic. They give rise to characteristic changes in electronic spectrum (*1*), vibrational spectrum (*2*), and nuclear magnetic resonance spectrum (*3*). These spectroscopic changes can only be understood if the electronic and vibrational wave functions of the hydrogen bonded partners are changed by the formation of the hydrogen bond. Further, there is no generally agreed way to partition the binding energy of atoms whose van der Waals radii overlap. Thus, the statement that the interaction is electrostatic because the binding energy can be mimicked with an electrostatic calculation containing arbitrary parameters is misleading. It is based on the wave functions of the interacting system, not the isolated reactants, and on a particular partitioning scheme. It should be noted that Gilli (*4*) has concluded that as "the O · · · O distance is shortened from 2.80 to 2.40 Å, the hydrogen bond is transformed from a dissymetrical O–H · · · O electrostatic interaction to a covalent and symmetrical O · · · H · · · O bond."

Warshel *et al.* argue that hydrogen bonds in an enzyme active site will be weak because of solvation effects, and imply that this site resembles a polar solvent. It is not easy to define the strength of a hydrogen bond. In the case of a covalent bond, the diatomic interaction between the bonded atoms is so much stronger than all secondary interactions that the homolytic dissociation enthalpy provides a satisfactory measure of bond energy for most purposes. Hydrogen bonds are weaker, and their strength is sensitive to the donor-acceptor distance, as is repulsive energy. We propose the following definition for hydrogen bond strength in an enzyme: With the heavy atom geometry adjusted so that the enzyme can perform its catalytic function, the hydrogen bond energy is the increase in Gibbs free energy that would occur if the hydrogen bond were deleted.

With this definition in mind, we note that the pK_a (negative logarithm of the acidity constant) of 1,8-bis(diethylamino)-2,7-dimethoxynaphthalene is about 16.3 (*5*). That's more basic, by a factor of 10^{12}, than a simple analog with only one basic site. Converting that to free energy gives 16.5 kcal/mol, in aqueous solution. We are all agreed that the presence of water tends to attenuate hydrogen bond strength. Also, the hydrogen bond in question is between two nitrogens, which generally do not give hydrogen bonds as strong as those between oxygens. The hydrogen bonds in enzymes usually involve at least one oxygen, and they are often formed in a much less attenuating matrix than an aqueous solution. We think these observations support our statement that hydrogen bond strength in enzymes may reach 20 kcal/mol in favorable cases. We do not dispute that a good deal of this can be mimicked by an electrostatic calculation, especially if the dielectric constant and the interatomic distances are not exactly known, and can be suitably adjusted. However, the spectroscopic evidence cited above indicates that such a calculation misrepresents the physics. Further, it is generally agreed by enzymologists that an enzyme active site is not equivalent to aqueous solution, and that one important role of the conformation changes that set up catalysis is to squeeze most of the water out of the active site. Unlike a solvent, the enzyme-substrate interactions that Warshel *et al.* refer to can be evolved to stabilize a distributed charge as well as a localized charge.

Warshel *et al.* quote a recent paper [reference (*18*) in their comment] as indicating that a very short hydrogen bond in an enzyme-inhibitor complex is not a strong one. This is a misinterpretation of the data. First, the measured dissociation constant of the inhibitor was not extrapolated to a pH where the inhibitor is protonated, and this must be done to get the true dissociation constant (the inhibitor binds with its carboxyl protonated). Second, there is no way to determine the degree to which the changes from substrate to inhibitor have *decreased* affinity. If allowance were made for such effects, the low barrier hydrogen bond that appears to be present would be producing a large amount of binding energy.

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Response: Tobin, Whitt, and I did not state in our report that hydrogen bond stabilization corresponding to 30 kcal/mol is realized in catalysis by serine proteases. We raised the question of how strong the LBHB between His^{57} and Asp^{102} is, and we specifically excluded 30 kcal/mol. Further, we pointed out that the decrease in rate upon disruption of the LBHB corresponds to 10^4 to 10^5, or 5 to 7 kcal/mol in activation energy. This is an important amount of catalysis. The statement by Warshel *et al.* that all hydrogen bonding effects in condensed phases are weak electrostatic effects is not supported by experimental observations.

1) Microenvironments at enzymatic active sites.

Warshel *et al.* state that hydrogen bonds in condensed phases are strictly weakly electrostatic in nature and that there are no strong hydrogen bonds. Their computational models for enzymes assign an essentially liquid solvent state to active sites. This is an arbitrary assumption. Evidence that substrate molecules are desolvated at active sites is overwhelming. The nonliquid nature of enzyme surfaces is evidenced most simply by the presence of hundreds of fixed water molecules at the water-protein interface of any enzyme. Bulk water is generally excluded from the interiors of enzymes, where the packing densities are 0.7 to 0.8, or approximately

that of a solid (*1*). Vacant active sites are packed slightly less densely (0.6 to 0.7), but are much more densely packed than liquids (0.4 to 0.5), and active sites that are filled by substrates or reaction intermediates are more densely packed. Many types of enzymes exlude water from their substrates as a prerequisite to effective catalysis. Examples include enzymes that protect radicals from quenching by water or enzymatic groups. Ribonucleotide reductase is an example that contains a stable tyrosine radical. Active sites generally have more in common with a solid state than a liquid state from the standpoint of the interactions of the substrate with enzymatic groups. It is incorrect simply to assign a liquid solvent state to an active site. Such an environment must be proven on a case-by-case basis.

To be useful, computational models must satisfy two tests: They must account for experimental observations, and they must predict the results of new experiments. Several experimental observations are not in accord with the idea that hydrogen bonds are strictly electrostatic or that they can not be stronger than about 5 kcal/mol.

2) Effects of hydrogen bonds on covalent bonds.

Polar covalent bonds are both covalent and ionic in nature, and there is no reason *a priori* to assign strictly electrostatic character to the weaker hydrogen bonds. In a recent paper, the lengths of covalent O–H bonds engaged in O–H · · · O hydrogen bonding were compared with the H · · · O distances in a large number of molecules (*2*). The dependencies of the covalent lengths were smoothly dependent on the separations of H · · · O and O · · · O, with no signs of discontinuities. The shorter the hydrogen bond the longer the covalent bond. The O · · · O distances ranged from 2.95 Å to 2.2 Å, and the H · · · O distances ranged from 1.2 Å to 2.0 Å.

3) Interactions of carboxylic acids with imidazoles.

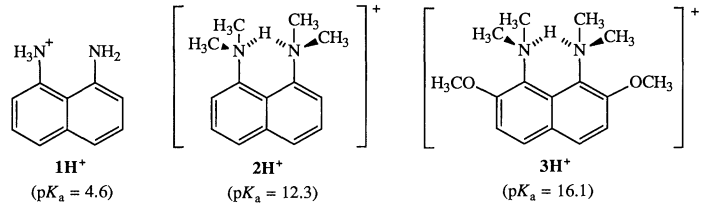
Warshel *et al.* state that the only complex between a carboxylic acid and the imidazole group is an ionic complex of the type $RCOO^-$----HIm^+. They base their conclusion on the results of coulombic computations that assign all stabilization to electrostatic attraction. They dispute the possibility that pK_a values of the peptides Asp and His can be similar in the active site environment. A small part of the difference between our views may be semantic; therefore, I will define terms. The term pK_a is generally used to place acidities on a scale. We did not say that the aqueous pK_a's of Asp and His are similar, only that their pK_a's in their respective microenvironments can be similar. The pK_a's of carboxylic acids are higher in organic solvents than in water. For example, the dissociation constants of carboxylic acids in water are generally 10^5 times higher than in ethanol (*3*). The acidities of positively charged acids such as imidazolium ions are much less solvent dependent. Matching of group pK_a's in enzymatic microenvironments does not refer to matching in aqueous solution. For a given group, we may refer to an *aqueous* pK_a and a *microenvironmental* pK_a, which may not be the same.

My students and I have studied the nuclear magnetic resonance properties of 1:1 complexes formed between carboxylic acids ranging in aqueous pK_a from 0.23 to 4.76 with *N*-methyl-imidazole dissolved in $CDCl_3$, CD_2Cl_2, or C_6D_6 (*4*). A plot of chemical shift for the acidic proton on the ordinate against aqueous pK_a on the abscissa reveals a biphasic relationship. The slope is positive from aqueous pK_a 0.23 to 2.2 and negative from aqueous pK_a 2.2 to 4.76, and the maximum value of the chemical shift is 18.3 ppm. The chemical shifts for the 2-proton of *N*-methylimidazole in the same complexes range from 8.9 ppm for the strongest acid to 7.6 ppm for the weakest, with the midpoint value of 8.2 ppm corresponding to an aqueous pK_a of 2.2. These compare with 8.9 ppm for *N*-methylimidazolium *p*-toluenesulfonate and 7.4 ppm for *N*-methylimidazole itself. For carboxylic aqueous pK_a's above 2.2, the complexes are increasingly nonionic and the acidic protons are increasingly shielded as the aqueous pK_a of the carboxylic acid increases. For acids with aqueous pK_a's below 2.2, the complexes are increasingly ionic and the acidic protons are increasingly shielded as the pK_a of the carboxylic acid decreases. The minimum shielding of the acidic proton occurs in *N*-methyl-imidazole complexes formed by carboxylic acids exhibiting aqueous pK_a's of 2.2. This is about 4.8 pK_a units lower than the aqueous pK_a of *N*-methylimidazolium ion, and this difference corresponds to the expected differential solvent effects on the aqueous pK_a's of the two species. That is, in the organic solvents the acidity, or microenvironmental pK_a, of a carboxylic acid is matched with that of *N*-methylimidazolium when the aqueous pK_a of the acid is 2.2. The chemical shift differences likely represent differential hydrogen bond strengths, and the strongest hydrogen bond in this system is probably between *N*-methylimidazole and a carboxylic acid with an aqueous pK_a of 2.2. These experiments also show that the pK_a's of ordinary carboxylic acids are not decreased by complexation with *N*-methylimidazole and that they do not necessarily form ionic complexes. For example, the complex between acetic acid and imidazole in organic solvents is nonionic and incorporates a weak hydrogen bond.

These results are similar to those reported by other groups for complexation between pyridines and carboxylic acids. The results of nuclear magnetic resonance and infrared spectroscopic studies are reviewed by Dega-Szafran and Szafran (*5*).

4) Strengths of hydrogen bonds.

A principal contention of Warshel *et al.* is that all hydrogen bonds in condensed states are similar and weak, ~5 kcal/mol. This view does not explain several spectroscopic and chemical observations. The pK_a values in aqueous solutions of the compounds $1H^+$ to $3H^+$ range from 4.6 to 16.1 (*6*, *7*). Thus, compound 3 is a stronger base than hydroxide ion. The pK_a of $1H^+$ is unexceptional and slightly higher than that of anilinium ion, that is, much lower than an ordinary primary alkylammonium ion because of resonance. The pK_a of $2H^+$ is higher than that of an ordinary tertiary ammonium ion. Steric inhibition of resonance owing to the bulky methyl groups could elevate the pK_a to perhaps that of allyl dimethylammonium ion, which is 8.79 (*8*), but not to 12.3. The pK_a of 16.1 for $3H^+$ is even higher and makes it a stronger base than the hydroxide ion. Because of steric inhibition of resonance, the methoxyl substituents should not be acid weakening. Indeed, their inductive electron withdrawing effects should make them acid strengthening. Nevertheless, the pK_a is 16.1. Therefore, $3H^+$ must be stabilized internally by some means to the extent of *at least* 10 kcal/mol relative to the allyl dimethylammonium ion. Also, simple tertiary ammonium ions such as allyl dimethylammonium ion derive some 5 kcal/ mol of stabilization from hydrogen bond donation to water, which is impossible for $3H^+$ owing to steric crowding. Loss of stabilization through hydrogen bond donation to water must be overcome by internal stabilization in $3H^+$, so we must add 5 kcal/mol to the 10 kcal/mol. This gives an estimate of about 15 kcal/mol for internal stabilization in $3H^+$. Hibbert and Emsley suggest the strong internal hydrogen bond as the source of this stabilization (*9*). If strong hydrogen bond-



ing is not the source of internal stabilization for $3H^+$, we will have to invent a new physicochemical phenomenon to explain the basicity of compound 3.

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$p34^{cdc2}$ and Apoptosis

Recently, Lianfa Shi *et al.* (*1*) reported on the involvement $p34^{cdc2}$ in two instances of apoptotic cell death. As apoptosis exhibits several features reminiscent of mitosis, it has been suggested that some of the cell cycle components that drive cells into mitosis may also be responsible for triggering apoptosis. The serine-threonine kinase, $p34^{cdc2}$, is essential for entry into mitosis and, if prematurely activated, can induce a process resembling apoptosis that has been termed "mitotic catastrophe" (*2*).

Fragmentin-2, a cytotoxic cell granule serine protease, can trigger apoptosis in cells exposed to this protease in combination with perforin, a pore-forming cytotoxic granule protein (*3*). Apoptosis induced by fragmentin-2 and perforin was shown by Shi *et al.* to be accompanied by a dramatic increase in $p34^{cdc2}$ kinase activity and to be inhibited by a $p34^{cdc2}$ substrate peptide (*1*). Also, FT-210 cells, which carry a temperature-sensitive $p34^{cdc2}$ that prematurely degrades at 39°C (*4*), resisted apoptosis in the presence of fragmentin-2 and perforin or staurosporine after pre-incubation at this temperature (*1*). These and other recent observations (*5*) suggest that $p34^{cdc2}$ may play a key role in several forms of apoptosis.

To explore whether $p34^{cdc2}$ activity is a general requirement for apoptosis, we used the FT-210 cell line, in combination with a diverse array of apoptosis-inducing stimuli, to ask whether these cells generally died less rapidly after culture at the restrictive temperature of 39°C ($p34^{cdc2}$ degraded), as compared with the kinetics of cell death after culture at 32°C (normal $p34^{cdc2}$ content).

FT-210 cells degraded their $p34^{cdc2}$, but not another cell cycle–associated kinase ($p33^{cdk2}$), when incubated at 39°C versus 32°C (Fig. 1), as previously reported (*4*). No changes in amounts of Cdc2 were detected in parental FM-3A cells under the same conditions (Fig. 1). Decreases in $p34^{cdc2}$ were also reflected in changes to the cell cycle profile of FT-210 cells incubated at 39°C, as these cells could not traverse the G_2/M boundary and arrested in late G_2 (*6*), whereas FM-3A cells traversed the cell cycle at either temperature (*6*).

Actinomycin D (Act D; an RNA synthesis inhibitor), hydrogen peroxide (H_2O_2), ultraviolet (UV) B radiation, VP-16 (a topoisomerase II inhibitor), cycloheximide (Chx; an inhibitor of protein synthesis), and C_2-ceramide (a sphingolipid implicated as a second messenger in apoptosis induced by TNFR and Fas ligation, as well as other forms of apoptosis) are all potent inducers of apoptosis (*7*). FT-210 cells, pre-incubated for 18 to 24 hours at 32°C or 39°C, were exposed to a range of concentrations of each agent. To minimize potential kinetic differences between the rate of apoptosis at 32°C versus 39°C, exposure to the various agents was conducted at 37°C (*1*). Cell death was quantitated 18 to 24 hours later.

Rather than being resistant to the induction of apoptosis after pre-incubation under conditions that degraded $p34^{cdc2}$, FT-210 cells proved to be equally susceptible to several of the apoptosis-inducing stimuli tested, whether pre-incubated at 32°C or 39°C (Fig. 2). In each case, the cell deaths observed were accompanied by typical features of apoptosis, such as condensation and fragmentation of the nucleus as well as DNA cleavage (*6*). G2-arrested FT-210 cells actually proved to be more susceptible to undergoing apoptosis in response to UV irradiation and H_2O_2, but the reasons for this are unknown.

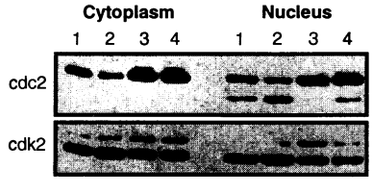

Fig. 1. Western blot detection of $p34^{cdc2}$ (top panel) and $p33^{cdk2}$ expression in FT-210 (columns 1 and 2) versus FM-3A cells (columns 3 and 4) after a 24-hour incubation at the permissive (32°C; columns 1 and 3) or restrictive (39°C; columns 2 and 4) temperature.

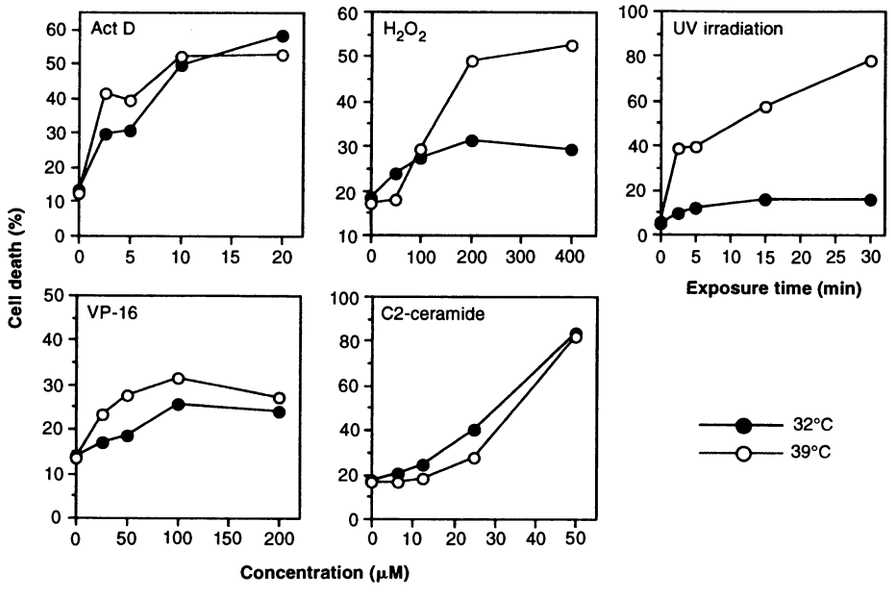

Fig. 2. Cell viability, as assessed by PI dye uptake (*8*) of FT-210 cells pre-incubated at either the permissive or restrictive temperature, followed by a further 18-hour incubation (at 37°C) in the presence or absence of the indicated stimuli. For UV B irradiation, culture dishes were placed on a 302 nM UV transilluminator and were irradiated from below for the indicated periods of time, as previously described (*7*). Each data point is derived from counts performed on 5000 cells. All treatments were carried out in triplicate. Results shown are representative of six independent experiments.