Pressure inside Small Cavities: A Possible Factor in Enzyme Catalysis and Selectivity

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Dedicated to Professor *Jack D. Dunitz* on the occasion of his 80th birthday

There are many different examples where atoms or molecules are found in cavities within larger (cage) molecules. The term host–guest chemistry has been used to describe this situation. This paper considers the possibility that pressure within the cavities can be measured or calculated in several possible ways. The various values for pressure for different systems can serve to compare them. It is suggested that pressure might be related to the rate of escape of the contents through an opening in the cage wall and to the equilibrium between inside and outside. Furthermore, in the case of substrates within enzyme active sites, it is suggested that the pressure is anisotropic. Pressure localized along certain directions may play an important role in the catalytic function of the enzyme.

When we have gas inside a container, we ordinarily think of its pressure. Is pressure still a useful concept when we have a single molecule inside another molecule? We can consider two categories of this situation. The fullerenes are closed molecular containers with very high barriers for the introduction or escape of guests. In contrast, enzymes have pockets that enclose one or more substrates, but they can readily enter and leave.

How do we get the pressure of gas in a container? We might begin by considering the ideal gas law: $PV = nRT$. We simply need the volume of the cavity and the number of molecules contained. On a more fundamental level, we might go back to the definition of pressure as the force a fluid exerts on a segment of the wall of a container per unit area. At the microscopic level, the force is the time-averaged force exerted on the walls resulting from collisions of molecules. This idea can still be applied when the container becomes smaller and contains less gas. However, when the quantity of gas becomes extremely small, pressure is no longer a continuous function. We have the pressure due to a single molecule inside a container, or two molecules, but never 1.5 molecules. Zero molecules will exert zero pressure. Pressure inside a molecular container should not be confused with 'internal pressure'. This term has long been used to describe a property of pure liquids associated with intermolecular attraction and repulsion [1].

With a large container, deviations from the ideal gas law occur because of interactions between the gas molecules. With a single gas molecule, there are no such interactions. However, when the atom or molecule inside the cavity occupies an appreciable fraction of the total cavity volume, the effective volume we should use in obtaining the pressure from the ideal gas law is the difference between these two volumes. We might call this the free volume. A large atom within a cavity cannot be moved to as many different positions as a smaller atom in the same cavity and will, therefore, exert a higher pressure on the walls.

Thus far, we have considered hard-sphere models. We have assumed that the volumes of the cavity and of the contents are well-defined. If we replace this hard-sphere model with one with a more realistic potential, the boundaries of the cavity and of the contents are no longer precisely defined. The ideal gas law no longer applies. In principle, we could still calculate the time-averaged force exerted by the contents on the walls and get the pressure. This can be a more-complicated calculation. One might want to do a molecular-mechanics simulation. In some cases, the flexibility of the container might have to be considered. It would be nice to have a general method for accurately calculating the pressure in all cases. However, in its absence, approximate formulas that apply under limiting circumstances may still be of interest.

A different approach for estimating the pressure of a molecule inside a container does not require knowing the volume. Suppose that we have a container where there is an opening in the wall big enough for a contained molecule to escape. It is reasonable to expect that the rate of escape (with vacuum outside) will be proportional to the pressure inside. The rate of gas entering an empty container through the same hole would be proportional to the external pressure with the same proportionality constant. At equilibrium, the rate of entry into empty containers will be equal to the rate of escape from full cavities. This is true, in principle, even in cases like He inside fullerenes, where the barrier to escape is very high, and both in and out rates are immeasurably low.

For this model, these assumptions lead to the following relations:

$$\text{Pressure inside} \bullet [\text{full}] = \text{Pressure outside} \bullet [\text{empty}]$$

$$\text{Pressure inside} = \text{Pressure outside} \bullet [\text{empty}]/[\text{full}]$$

In many cases, one can experimentally measure the ratio of empty to full containers as a function of outside pressure to get this effective pressure inside, which is also the equilibrium constant. The same equation can be obtained in another way. Suppose that we have an array of small (but not yet microscopic) chambers each communicating with a large chamber. The small chambers will attain the pressure of the large chamber at equilibrium. If we shrink these small chambers, the number of atoms they contain will decrease while the pressure in them stays the same. Eventually, many of the small chambers will contain no atoms. To make the average pressure inside them remain equal to the pressure in the large chamber, the pressure in the occupied chambers must be greater than this pressure. Again, the pressure in a single occupied small chamber will be related to the ratio of numbers of occupied and empty small chambers and the pressure in the large chamber.

This method of estimating the pressure inside does not require a hard-sphere model for interactions between the walls and the contents. Suppose that we turn on an attractive *Van der Waals* interaction of the atom inside with the walls of the small chambers. An Ar-atom inside C_{60} might be an example. There will be a net flow of gas in. The fraction of containers occupied will increase, and our formula will indicate a lower pressure inside. The stronger the interaction, the greater will be the decrease in the pressure estimated this way. Instead of a general attractive interaction, we could also consider adding discrete binding sites inside the small chambers. The total amount of gas in them will increase, giving us a lower value for the pressure inside.

R. J. Cross and I have studied noble gas atoms and small molecules within fullerene cages [2–9] and have thought about the pressure inside. We estimate that the pressure of one He-atom inside C_{60} is *ca.* 2,000 atm at room temperature using the ideal gas law. This high pressure is not a result of the walls 'squeezing' the He-atom. There is plenty of room inside for the He-atom and the walls of the fullerene interact little with it. In fact, we have observed [10][11] two He-atoms inside C_{60} and C_{70}. A simple way of looking at the situation is that the average volume available to the He-atom in the gas at 1 atm pressure is reduced by a factor of *ca.* 2,000 when the He-atom is put inside C_{60}. The He-atom, with a Maxwellian distribution of kinetic energies appropriate to the temperature, undergoes a very large number of collisions with the walls, and, therefore, the walls experience high pressure. Going from C_{60} to the much smaller dodecahedrane cavity [12], the estimated pressure goes up to roughly 10^{35} atm. For this case, the model of a freely moving He-atom colliding with the walls is clearly inappropriate. There is a strong repulsive interaction of the He-atom with all the C-atoms at once, leading directly to the enormous pressure. The motion of the He-atom has the character of vibration in a steep-sided well.

It is interesting to consider other situations where the concept of the pressure of an atom or molecule inside a cage might be applied. Over fifty years ago, *Powell* coined the term clathrate for crystals where the lattice of a major component had cavities large enough to hold atoms and small molecules. Much of his work involved quinol (hydroquinone). Quinol normally forms the α-crystal. However, in the presence of some small molecules or atoms, the β-modification was formed. In it, there were cavities large enough to hold small guests. In 1949 and 1950, *Powell* described quinol clathrates of Ar, Kr, and Xe [13]. These were formed under pressures of tens of atmospheres of the gases. Clathrates of He were also tried without success. The He probably escaped from the crystal. *Powell* found that the cavities were incompletely occupied. He estimated a volume of the 280 cc/mol for the cavity from the crystal structure and estimated the pressure of Ar inside of 91 atm (probably from the ideal gas law). I would like to thank *Jack Dunitz* for pointing out this work to me. He has suggested that fullerenes containing noble gas atoms might be called molecular clathrates.

Now let us consider a case that seems far removed from noble gas atoms inside fullerenes. Consider a substrate inside the catalytic pocket of an enzyme. Substrates fit fairly closely. Might they not be at high effective pressure? If they are at high pressure, might this not affect the rate of the reaction? It is well-known that reaction rates can be affected by pressure [14–16]. The conventional way of dealing with this is to consider that the transition state may occupy a different volume than the starting material. We talk about a ΔV of activation. If it is negative, the rate of the reaction is accelerated by higher pressure. Higher effective pressure on the substrate within the cavity of the enzyme could, therefore, be a factor in increasing the reaction rate. An acyclic starting material going to a cyclic transition state might be associated with a substantial negative ΔV. When two substrates react with each other inside the enzyme active site, the ΔV for the reaction is also a likely factor. In general, the two substrates come closer together at the transition state, and ΔV is negative.

If the pressure of the substrate within an enzyme – substrate complex were extremely high, why wouldn't the substrate escape? In fact, if there were a very high pressure of a substrate in an enzyme-binding pocket, the equilibrium amount bound would be very small, as one can conclude from the discussion above. The reaction rate in the enzyme – substrate complex might be accelerated due to the high pressure, but this would be compensated for by a much lower occupation of the enzyme site. However, to stabilize the enzyme – substrate complex and make its equilibrium population higher, there are groups on enzymes that interact with the substrate. There may be H-bonding, charge-interaction, or π-stacking, or just *Van der Waals* forces. These attractive interactions will, in general, lower the pressure inside and increase the fraction of enzyme cavities occupied. On the other hand, if the 'wrong' substrate tries to enter an enzyme pocket, it would exert enormous pressure because it does not fit and/or the favorable interactions are not present. The occupation fraction would, therefore, be extremely low. Enormous pressure when the 'wrong' substrate enters the binding site is another language one could use, which is related to the old lock and key theory used to account for the selectivity of enzymes.

Enzymes generally operate in solution. For a substrate dissolved in solution, we have to consider the solvation forces and energies. To avoid worrying about the details of solvation, we can consider the vapor pressure of the substrate above the solution. This vapor is in equilibrium with substrate dissolved in the solution. The solution is, in turn, assumed to be in equilibrium with the substrate bound in the enzyme pocket. Whether or not we can actually measure this vapor pressure, considering it allows us to think in terms of the equilibrium between the enzyme – substrate complex and gaseous substrate. We can use the common language of pressure in both cases.

There is a very important way in which enzyme – substrate complexes are different from He inside C_{60}. A gas within a closed container has a single value for the pressure. Pressure on any part of the wall is the same. The pressure is isotropic. The situation is different for an enzyme – substrate complex. A substrate within an enzyme catalytic pocket is almost certainly bound in a single orientation, and, as a result, the pressure can be anisotropic. The match between the size of the substrate and the size of the pocket will, in general, be different in different directions. Also, there are likely to be attractive forces (H-bonding, charge interaction) in some directions that lower the pressure in these directions. We might, therefore, plot a contour map for substrate pressure for points on the surface of the cavity.

Consider a simple hypothetical model. Suppose that we have a cylindrical cavity closed at both ends. Now imagine that there is a cylindrical molecule, which fits inside the cavity. It can slide back and forth along the length of the cylinder but cannot turn around. It can also move back and forth and hit the sides. The molecule will have a distribution of velocities along the axis of the cylinder and will frequently hit the ends, exerting a pressure. Making the cylinder shorter will increase the number of these collisions and, therefore, raise the pressure on points on the end faces. The molecule will also be moving at right angles to the cylinder direction and will, therefore, exert pressure on the sidewalls. Making the cylinder fatter or thinner will affect the pressure on the sidewalls but will affect pressure on the ends less. The pressure, defined by the average force exerted by collisions per unit area, is different on the ends and the sidewalls.

In an enzyme–substrate complex with no symmetry, the pressure exerted on the walls could be different at each different point of the cavity surface. In general, the transition state for a reaction will have a different shape than the substrate. As one goes to the transition state, the species is likely to become larger in certain directions and smaller in other directions. Therefore, there is no longer a single overall value for ΔV. It may be positive in some directions and negative in others. Part of the catalytic effect of the enzyme might be that it is designed to squeeze more on the substrate in a direction that will contract and less on parts of the substrate that will expand on going to the transition state. If there were two possible different reactions, the shape of the pocket alone might discriminate between them, favoring one and disfavoring the other.

Consider a reaction between two substrates. In general, the enzyme will bind the substrates so that the groups that are going to react are facing one another and aligned in the correct relative orientation. The groups on the enzyme that interact with the substrates and make binding in the pocket more favorable might well act in an anisotropic fashion. It could happen that most of these attractive interactions are at right angles to the vector between the centers of the two reactants. If there were no groups binding the substrates from the sides opposite to the reaction face, the force along this vector due to pressure from the ends would not be reduced from what it might be in a completely isotropic situation and might be very high.

In the following *Figure*, the red areas represent high pressure and the green bands signify attractive interactions such as H-bonds. These lower the pressure in those directions.

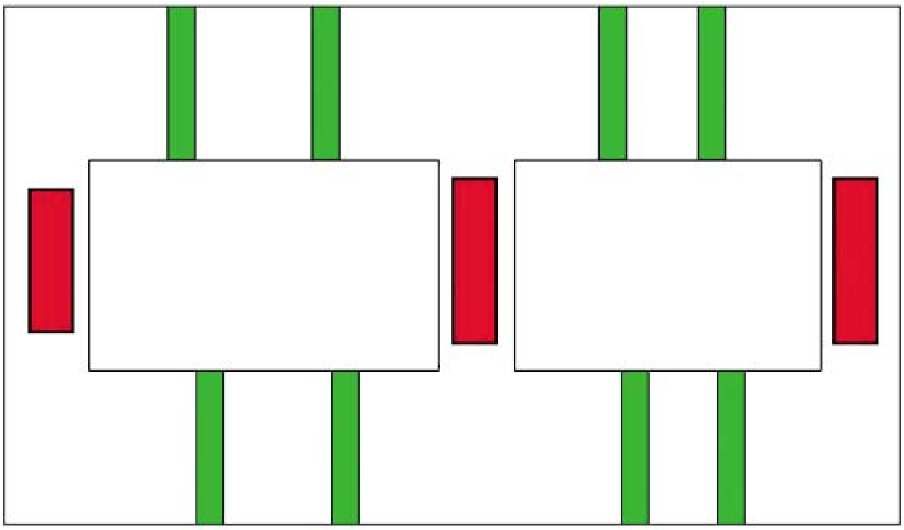

Fig. 1.

We might think of the enzyme pocket constraining the molecules within a linear space instead of a volume in three dimensions. There would be a greatly increased number of collisions between two molecules corresponding to a very high pressure in this direction. Only the regions of the substrates that would lead to reaction collide in the necessary relative orientation. In contrast, in solution in the absence of the enzyme, there will be many fewer total collisions between the reactants. Most of the collisions that occur cannot lead to reaction because the reactive groups do not hit in the required orientation.

One could go from a model considering the substrate as a solid object to a more-detailed one where one considers the atoms specifically. One would then naturally switch from considering pressure on the surface to the vector sum force on each atom. There are some constraints on the $3N$ components of these forces. The sums of the x, y, and z components on the atoms of a substrate must be zero or the substrate would move. In a similar manner, the torques around the three axes (two for a linear substrate) must be zero or the substrate would start rotating. We can think about the forces on the atoms of a transition state in the pocket. The changes in these forces on going to the transition state will be related to the catalytic effect of the enzyme.

The concept that the pressure in different directions resulting from the limited volume or the limited room for the molecules to move is an important factor in enzyme catalysis is worth considering. It certainly is not the only factor but is one that has not been generally discussed. It should be recognized that pressure is related to entropy. The entropy of a quantity of an ideal monatomic gas in a container is a simple function of the pressure. However, in more-complicated systems, entropy includes many other terms. The concepts of pressure and force are easier to grasp intuitively and to consider separately from other factors. They might also be the basis of detailed calculations to explore the possibility of their effects on enzyme-catalyzed reactions.

It is interesting to think about pressure inside other kinds of molecular enclosures. Atoms and molecules enter and reside in cavities of xeolites. Some of them have cavities large enough to hold several ^{129}Xe atoms. The Xe-NMR spectrum shows separate peaks for chambers with one, two, three, *etc.* atoms. The intensities should yield the pressures for these situations. The ^{3}He-atoms can be seen, with He-NMR, inside solid polymers and within molecular crystals [17]. Again, it may be useful to consider the pressure of He in the cavities in these materials.

Finally, one might also consider the model of an atom inside a cage when considering a crystal of a single pure substance. For example, He, which crystallizes in cubic space groups, has 12 nearest-neighbor atoms surrounding each He-atom. One might regard these 12 neighbors as a cage containing a single He-atom. If one neglects the effects of non-nearest-neighbor interactions and surface effects, the pressure in this cage should be equal to the external pressure. He is unique in that, even at absolute zero, it does not crystallize. To get crystals of He, one must apply an external pressure substantially greater than 1 atm. One can imply from this that all conceivable crystal structures have pressures of He inside greater than 1 atm. *Jack Dunitz* has informed me that, at 50 atm, He crystallizes in the hexagonal close-packed structure. The lattice parameters yield a *Van der Waals* radius of 1.8 Å. At 1200 atm, the structure changes to a cubic close-packed lattice yielding a *Van der Waals* radius of 1.5 Å. If a series of crystal structures were obtained at different pressures and temperatures, the volumes of the cages could be obtained from the measured lattice parameters. One could interpret this data as a series of points on the equation of state for He in the cage of its nearest neighbors.

The concept of the pressure of a gas in a container of molecular dimensions has been discussed for a variety of different situations. No single method of obtaining the pressure is found to be applicable to all cases. Different methods of approximation are suggested. Nevertheless, the values obtained are interesting and might be useful for comparative purposes.

Temperature is another important widely used variable, even though there is no simple universal definition of it or way of calibrating it from first principles. *Maxwell* [18] defines temperature by giving detailed instructions for making a Hg thermometer and calibrating it at the ice point and boiling point of water. He also describes an '*air thermometer*' based on assuming that air is an ideal gas. It is easier to define temperature on a differential basis. Heat will flow from a hotter body to a cooler body when they are in contact. In a similar manner, gas would flow from a chamber at higher pressure to one at lower pressure, if they were connected.

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