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Biogenic Ice Nucleation: Could it be Metabolically Initiated?

GERALD CAPLE,† DAVID C. SANDS,‡ R. GARY LAYTON,§
WILLIAM V. ZUCKER† AND J. R. SNIDER†

† *Department of Chemistry, Northern Arizona University, Flagstaff, AZ* 86011, *U.S.A.*; ‡ *Department of Plant Pathology, Montana State University, Bozeman, MT* 59717, *U.S.A.*; § *Department of Physics, Northern Arizona University, Flagstaff, AZ* 86011, *U.S.A.*

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Two species of bacteria, *Pseudomonas syringae* and *Erwinia herbicola* can initiate warm temperature (−1° to −3°C) ice nucleation events. This ice nucleation could be important in atmospheric ice nucleation processes. These bacteria are commonly found on plant surfaces and are responsible for initiating the ice nucleation events that lead to frost damage in plants. The mechanism of this ice nucleation event is unknown. Using assumptions, theoretical calculations, and the known metabolic requirements for these bacteria, we develop the idea that endergonic metabolism can be the basis for the initiation of an ice nucleation event. We speculate that altered oxidative metabolism of these organisms, lack of cytochrome *c* for example, could be an important factor. A purely thermal effect leading to ice nucleation is unlikely, but the possibility of a metabolically driven conformational change which may involve a redistribution of ionic charge leading to an exceptionally good ice nucleating site is one way of explaining the properties of these bacteria. A gel collapse may be involved, removing water from an "unfreezable state" and making it available for the critical embryonic ice crystal.

Introduction

The number of water molecules required to form a stable particle of ice is sufficiently large that the probability for the unaided (homogeneous) nucleation of an ice crystal is negligible above −40°C. Thus water melts at 0°C, but freezes at a temperature which depends on the freezing nuclei present in the particular water sample. In the absence of an ice nucleation initiator, microdroplets of pure water can be supercooled to −40°C without conversion to ice crystals (Pruppacher & Klett, 1978). Even for the well studied freezing nucleus, such as silver iodide, much uncertainty still exists as to the exact nature of the nucleation event, but it is known that nucleation occurs only at specific active sites whose nature is only partly understood.

Two species of bacteria, *Pseudomonas syringae* and *Erwinia herbicola* initiate ice nucleation at temperatures as high as −1°C relative to the bacteria-free medium which freezes at temperatures 10° to 15° lower (Maki *et al.*, 1974). These bacteria are well known plant pathogens that are common on plant surfaces (Maki & Willoughby, 1978). Freezing events initiated by these bacteria under field conditions are partially or wholly responsible (Arny *et al.*, 1976; Lindow *et al.*, 1978*a*,*b*; Marcellos & Single, 1976, 1979; Anderson *et al.*, 1982) for the approximately 14

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billion dollars of crop losses annually. In the absence of an ice nucleation initiation, many common crop plants, including fruits and vegetables, can survice light frosts with temperatures as low as −6°C, frequently saving the entire crop (Lindow *et al.*, 1978*c*). Sands *et al.* (1981) and Jayaweera & Flanagan (1982) have shown that *Pseudomonas syringae* are also found in the atmosphere, or associated with rain drops, and have suggested that bacterially induced ice nucleation events may be involved in atmospheric precipitation processes. Clearly, the mechanism(s) underlying these processes are of great importance. In this paper, we wish to propose that endergonic metabolism could drive the bacterially induced, ice nucleation process. The necessary assumptions and requirements for this proposal are discussed.

Bacterial Induced Ice Nucleation

SITES

Maki *et al.* (1976) suggested that there is a single ice nucleus active site on each cell. Repeated drop freezing experiments have shown that that this ice nucleus active site is not a static or permanent site or event (Maki *et al.*, 1976; Lindow *et al.*, 1982*a*). The experimental evidence supporting the statement that the ice nucleation site is not static is that droplets containing cells do not freeze in the same order upon repetitive freezing experiments, if the freezing events were caused by a permanent site, the droplets would *freeze* in the same order at the same temperature. This can be taken as evidence that different ice nucleus active cells initiated the freezing event. Potential ice sites appear to exist in all the cells, but are only occasionally expressed at warm temperatures. This implies a dynamic event is taking place at the site to cause ice nucleation initiation. These workers also reported that there are two different ways these ice nucleation sites might be active. One of them, the "warm site" is active at −1° to −5°; the other "cold site" is active at −8° to −12°. The cold temperature site was shown by Maki *et al.* (1974) to survive cell disintegration, and ice nucleation events occurring at temperatures warmer than −5°C require a normal complete cell. Lindow (1982*b*) has recently demonstrated that both sites are localized in the outer cell membrane and that an ice nucleation active site is converted to a non-active site under certain conditions with first order kinetics. Cloning experiments (Orser & Lindow, 1981) have shown that the ice nucleation site has a genetic basis and probably involves a protein. This information could imply that the two sites are either contiguous or localized to identical regions of the membrane and that discrete events distinguish their separate activities.

INHIBITION AND POTENTIATION OF SITES

Schnell (1974) and Vali *et al.* (1976) determined that when the bacteria are deprived of oxygen, only the warm site disappeared. Literally, these experiments show that while the total number of sites do not change, the warm temperature freezing events can be turned off and on at will. On the basis of these findings,

Schnell (1974) implicated discrete metabolic events in the warm temperature ice nucleation processes and that oxidative phosphorylation might be involved. Warm temperature ice nucleation is suppressed by treatment with respiratory inhibitors and with reactive chemicals such as borates, urea, high or low pH (Kozloff, 1983; Yankofsky, 1981; Lindow, 1982*a*,*b*). These data suggested that it is only the warm temperature ice nucleation event which can be metabolically induced, where something most occur at the site to induce nucleation, while the cold temperature site appears to be independent of metabolism. In support of this idea, we have recently shown (Caple *et al.*, 1982) that the addition of galactose to cultures of *Pseudomonas syringae* increases the number of warm temperature ice nucleation sites dramatically over the course of 3 hours. Anderson *et al.* (1982) reported this same effect upon the addition of glucose. Growth and plating studies show that this is activation of existing sites, not the creation of new sites on cells. We speculate that the warm ice nucleation event has energetic requirements that can be met for some reason by a concurrent metabolic process. Galactose and glucose are common metabolizable sugar substrates, suggesting that discrete metabolic events may be necessary for the formation, integrity, and expression of the warm site. In this respect, it is interesting to note that galactose also serves as a major sugar constituent in the "anti-freeze glycoprotein" in the circulatory system of arctic fish (Yeh & Feeney, 1978).

METABOLISM: GENERAL CONSIDERATION

Oxidizable substrates provide the necessary energy input enabling an organism to carry out many endergonic reactions, both catabolic and anabolic in nature. The overall efficiency of energy conversion for oxidizable substrate (glucose, for example) to the typical energy currencies of the metabolizing cell (ATP, PEP, NADH, etc.) can be calculated and figures of 40% have been cited, using reasonable assumptions concerning pool metabolite concentrations, temperature, etc. (Lehninger, 1982). The free energy not converted to energy currencies is, of course, lost to heat. At the extremes, certain cryophilic and thermophilic bacteria are able to metabolize and multiply at temperatures from a low of near 0°C to as high as 250°C (Baross & Deming, 1983). In the case of the ice nucleating active bacteria *Pseudomonas* and *Erwinia*, the unique ability of these plant pathogens to induce ice nucleation on plants and possibly in the atmosphere may have been a positive selection factor for the evolution of these traits. Leaving aside such teleological considerations, however, and more to the point: could a bacterium use the energy draining steps in its metabolism, viz., its endergonic reactions, to advantage when temperature stress is a factor in its environment? There are many endergonic reactions in a cell (the first two steps in the catabolic degradation of glucose, oxidation of lipids, lipid synthesis, nucleic acid synthesis, protein synthesis, etc.); is it possible that these steps separately or in concert could be used by an organism to induce an ice nucleation event? The energy equivalents of many of these steps are large, often involving the hydrolysis of a single ATP to ADP, providing a net drain of 10–12 kcal/mole to three times that amount if an NADH or NADPH is consumed in the reaction. We will attempt to quantify this possibility, using reasonable assumptions.

THE FREEZING EVENT: GENERAL CONSIDERATIONS

The initiation of a freezing event can be described by classical nucleation theory (Zettlemoyer, 1969). This approach provides physical insight lacking in the more abstract versions of nucleation theory.

To apply this theory it is necessary to assume a shape for the critical ice embryo. The usual assumed shape is a spherical cap, but various cylindrical or polyhedral shapes have been used. Fortunately the quantitative results are not highly dependent on the specific shape assumed. The free energy of the nucleus is calculated in terms of its radius, the various interfacial free energies (surface tensions) and the volume free energy. Surface tension terms are positive and proportional to the embryo surface area (which is proportional to r^2). The free volume term is negative and proportional to r^3. Due to the energetic requirements of forming a surface, small assemblies of molecules are unstable with respect to the parent phase. Above a certain critical size (the parameters of which are discussed in Pruppacher & Klett (1978)), the volume term dominates and the addition of still another molecule causes a decrease in free energy, hence, the embryo grows spontaneously and becomes an ice crystal. The exact number of molecules required is not well known, but for the thermal cooling mechanism considered in this paper, an estimate of 250 molecules is realistic (Hale, 1981).

A consideration of the kinetics within a population of embryos allows the derivation of an expression for the rate of formation of critical embryos. For a given set of conditions, this rate is negligible above the threshold temperature and increases extremely rapidly below this temperature. Thus it is possible to speak in relatively exact terms of the temperature at which a particular nucleus is active in nucleating ice (Vali, 1971). Any substance which decreases it is an inhibitor.

While in theory many factors affect freezing temperatures, sensitivity to changes in the interfacial energies is so great that the other factors may be ignored. For details see Zettlemoyer (1969) or Pruppacher & Klett (1978). Thus we are left with three ways in which a site may become active at warmer temperatures. The bacteria may:

(1) Directly absorb heat from a small volume of water, lowering its temperature to the threshold temperature of the bacterial site.

(2) Effect a change in the ice-water interfacial energy. Such a change could occur if the nucleation occurred in a gel-like coating created by the bacteria. This is because gels undergo a critical change in milliseconds (Tanaka, 1981).

(3) Alter the ice-membrane interfacial energy. Changes in cell surface ionic distributions or conformational changes in constituents of the cell membrane could accomplish this.

Each of these mechanisms will now be considered in more detail.

FREEZING BY DIRECT COOLING

This unlikely mechanistic postulation concerns a metabolic cooling of the ice nucleating site, but it is necessary to discuss any effect which may be pertinent. To properly consider this mechanism we need to have some idea of the energy required,

the time involved, the energy available, and the thermal processes tending to keep the system in equilibrium. In a microscopic living system, the instantaneous temperature at the cell surface does not have to be identical to the temperature of the surrounding medium. Assuming heat conduction processes are slow compared to the time scale of the metabolic processes on or near the cell surface, areas of that cell surface could cool long enough to become potential sites for nucleation.

The freezing event to be discussed involves cooling the site from $-1°$ to $-7°$ (approximately), removing this energy, and inducing nucleation. It is assumed that the ice nucleation site is always active at $-7°$ or below, even if the mechanism is unknown. The energy required for this is $6{\cdot}5 \times 10^{-19}$ calories.

Energy to freeze 250 molecules of H_2O:

Heat of fusion ice $(H_2O) = 80$ calories/g

$T(H_2O)$ from $-1°$ to $-7° = 6$ calories/g H_2O

Total energy $(-1°$ to $-7°) = 86$ calories/g H_2O

$$\frac{86 \text{ cal}}{x \text{ cal}} = \frac{3{\cdot}34 \times 10^{22} \text{ molecules}}{2{\cdot}5 \times 10^{2} \text{ molecules}} \qquad \text{(number of molecules in 1 g } H_2O\text{)}$$

$$= 6{\cdot}5 \times 10^{-19} \text{ calories to freeze 250 molecules } H_2O.$$

The induction period (the elapsed time between an increase in super-cooling and an observable nucleation rate) is not well known and, as discussed, neither is the size of the critical nucleus. The choice is between unsatisfying theories that give numbers, and elegant theories which provide no numbers. If we use existing theories, however, tempered by a consideration of available data (Mason, 1969; Zettlemoyer, 1969), we can arrive at an estimate of the induction time of 10^{-5} seconds for a critical nucleus of 250 water molecules. Both should be at least of the correct order of magnitude. According to theory, neither quantity should change by more than 10% between $-1°$ and $-11°$ if all other quantities are held constant.

The energy available may be calculated by considering a specific metabolic process.

(a) The utilization of ATP in the first steps of glycolysis has the energy equivalent of $2{\cdot}5 \times 10^{-20}$ cal/molecule of glucose

$$\frac{15 \times 10^{3} \text{ calories/mole glucose}}{N} = 2{\cdot}5 \times 10^{-20} \text{ cal/molecule glucose}$$

(b) To induce freezing in 250 molecules of water, as described above, would require the metabolism of 27 molecules of glucose

$$\frac{6{\cdot}5 \times 10^{-19} \text{ calories/250 molecules } H_2O}{2{\cdot}5 \times 10^{-20} \text{ calories/molecule glucose}}$$

This is a minimum estimate of the energy required to induce ice nucleation since cooling an equal volume of cell mass may also be included. This however would only consume two more molecules of glucose, assuming a specific heat of 1 cal/g.

(c) Glucose is consumed by *Pseudomonas syringae* in solution at the rate of 1 g/hr 10^8 cells/ml at 23°. This is equivalent to $8{\cdot}6\times10^6$ molecules glucose/cell/second. Assuming a reasonable Q_{10} value of 2·2 (Stryer, 1981), the glucose consumption at −3·0°C (the temperature at which ice nucleation can occur) would be $8{\cdot}6\times10^5$ molecules per second for a single cell.

(d) Assuming that there are 10–100 enzymes for the endergonic reactions per cell, with turnover numbers between 10^3 to 10^4, it can be calculated that the time to metabolize 29 molecules of glucose would fall between $3{\cdot}2\times10^{-2}$ and $3{\cdot}2\times10^{-4}$ seconds. It will be assumed that any single metabolic event, of equivalent energy, could occur in as little as 10^{-5} seconds.

The heat transfer process is well understood. The freezing event discussed involves lowering the temperature from −1° to −7°C. Stephenson (1960)† has shown that no region in a 50 μm diameter sphere will differ in temperature from another volume element by more than 0·08°C when the system is being cooled at rates less than 10^4°C/min. The temperature differential is inversely proportional to the radius squared. The rod like bacterium *Pseudomonas syringae* has a minimum diameter of ~1 μm. It can be calculated that a cooling rate of $4{\cdot}3\times10^7$°C/sec would be necessary to affect a 6°C temperature change in *Pseudomonas syringae.* Thus to cool by 6° a site on a bacterium would involve a time approximately $1{\cdot}4\times10^{-7}$ sec. Since this is orders of magnitude faster than our calculations of any possible metabolic events permit, we consider it extremely unlikely that an ice nucleation site could be created by heat transfer processes alone. This is consistent with the fact that a living cell in distilled water can still nucleate (Anderson, 1982; Yankofsky, 1983).

FREEZING BY CHANGING ICE-WATER SURFACE

This mechanistic postulate concerns a metabolically created ice nucleation site. In this and the following mechanisms, the energy requirement is not so obvious, but it is still required. However, it is not necessary to impose very small critical ice nucleus of 250 water molecules. It is implicitly assumed in this discussion that energy currency replacement is rate-limiting, for whatever reason, and that ice formation occurs in times too short, about 10^{-5} sec as postulated earlier (see also Beall, 1983), for further metabolic events to override purely physical processes involved in ice nucleation events. In fact, it may be exactly this trait which distinguishes ice nucleating from non ice nucleating bacteria.

A characteristic property of the cell wall/outer membrane complex of many bacteria is their mucopolysaccharide, glycoprotein, and peptidoglycan surface. Apart from their possible functions on the cell surface, in the presence of water these polymers can take on the characteristics of a gel, and gels have properties which we believe lend themselves to understanding how ice nucleation sites may be activated. Tanaka (1981) has defined a gel as "... a form of matter intermediate between a solid and a liquid. It consists of polymers or long chain molecules, cross linked to create a tangled network in a liquid medium. The properties of the gel

† We wish to thank a reviewer for bringing this reference to our attention.

depend strongly on the interaction of these two components. The liquid prevents the polymer network from collapsing into a compact mass; the network prevents the liquid from flowing away." A balance of forces maintain this state of affairs; disturbing it even infinitesimally can bring on a phase transition and collapse the gel. The factors that affect gel phase transitions are well known and include pH, solvent, solute concentration, ionic strength, and temperature (Tanaka *et al.*, 1978). Water, tied up in the gel, has a high "non freezable fraction" (Beall, 1983) and thus would not be available to a critical ice nucleus at warm temperatures.

The theoretical aspects of gel collapse mechanism as a discrete biochemical event have been discussed by Wookey (1980). The author suggests that the metabolic requirements of such an event were linked to a metabolic event in the glycolysis pathway. An ATP/ADP ratio decrease could result in contraction of the mitochondria. Wookey (1980) also postulated that the Ca^{++} to phosphate ratio could act as the trigger. Small changes in the Ca^{++}/Mg^{++} ratio, as small as 25 namomolar can change the activity of an enzyme by a factor of 70.

Powell (1982) has also discussed the order–disorder changes in gluco polymers in biological systems. In many of the cases discussed, lowering the temperature causes an increase in the order of the system; it becomes more crystalline. The same effect can be observed upon the addition of divalent ions which can also cause an ordering effect. The only requirement here is that this change lead to the formation of an ice nucleating site.

Sandejas & Hudson (1968) have considered the case where nucleation takes place in thick adlayers and shown that considerable enhancement of ice nucleation is possible. Although the case they considered was for nucleation from the vapor, the similar geometry makes comparisons of trends, if not absolute numbers, valid. For our case we note that a small change in the physical or chemical characteristics of the layer in which the nucleation occurs can modify the ice/layer interfacial energy which will make a very large change in the freezing temperature. Thus the collapse of a gel or a change in its characteristics due to the temperature or ionic content could allow (or prevent) the sudden formation of an ice crystal on a bacterial surface with no change in the actual nucleating site itself. None of the few facts that are presently known disallow this possibility. The ionic collapse process which occurs at temperatures of −2°C would involve a metabolic step which takes place only in a living cell, while the temperature transition step occurs about −9°C and can involve sites in dead cells or parts of cells.

FREEZING BY CHANGING THE ICE-SITE INTERFACIAL ENERGY

This mechanistic postulate concerns actual ionic changes in the outer surface of the bacterial ice nucleating site.

Pseudomonas syringae is one of a few species of pseudomonads found to date that lacks cytochrome *c*; whereas an autooxidizable cytochrome *b* is always present (Sands *et al.*, 1969). This is also true for two other ice nucleating species *Erwinia herbicola* and *Xanthomonas campestris* p.v. *Transluceus* (Kim, 1984). Because of this, *P. syringae* will have an unusual oxidative metabolism which could certainly

affect the movement of ions and substrates across the membrane. Kosloff *et al.* (1983) has shown that ice nucleation is not dependent of pH change in the range 6–8·5, and it is therefore unlikely that proton disequilibrium could account for the ice nucleation event. However, a pH change at 6–8·5 is a small pH range and a localized effect cannot be ruled out. A change in the ionic strength, using polyvalent ions, would, however, be a strong candidate for the creation of an ice nucleation site on the bacterial surface or on the membrane. Divalent ions, Mg^{++} and Ca^{++} are typically involved in biological transport processes. In some instances, it is believed that these ions are obligatorily inplicated (Lehninger, 1975) in ATP-directed active transport. The influx and efflux across membranes of other ions is also known to be coupled to electron transport and oxidative phosphorylation.

We postulate that there is a perturbation in the ion distribution that leads to a rapid alteration of the bacterial surface energy. This perturbation is in response to some critical conditions of stress or temperature. Such a change would alter the threshold temperature of the site directly since it is known that changing ions or the charge at an ice nuclei active site modifies the ice nucleation properties of that site (Layton, 1973; Pruppacher & Pflaum, 1975). Additional trigger events leading to site activation could occur if the change in ion concentration resulted in a conformational change, which changes the hydrophobic-hydrophilic properties at the site, in one or more unique proteins, or if it led to a gel phase transition (Tanaka, 1981). If this charge is highly localized and in the time frame of 10^{-5} sec (Beall, 1983), because it is initiated only at a specific redox or phosphorylation site, then it and the ice nucleation event could happen, at least at the rate of a single metabolic step at that site. It is of course possible that both the gel collapse and change of ionic strength are a synergistic process, metabolically initiated, that produce the warm temperature ice nuclei active site. Although all INA bacteria appear to lack cytochrome *c*, many INA inactive strains of *Psudomonas syringae* also lack cytochrome *c*, thus a cytochrome absence may be necessary, but is not sufficient by itself to induce ice nucleation.

Conclusion

The origin of the ice nucleating site on *Pseudomonas syringae* which is active at temperatures just below the freezing point of water is a problem of great practical and scientific importance. We have considered or speculated on possible mechanism(s) for the creation of this site in light of nucleation theory. There are at least two mechanisms, which may be closely related, that are consistent with both nucleation theory and with the known properties of the bacteria. These mechanisms may also be related to the unusual metabolism of the INA bacteria.

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