Blocking rapid ice crystal growth through nonbasal plane adsorption of antifreeze proteins


Antifreeze proteins (AFPs) are a unique class of proteins that bind to growing ice crystal surfaces and arrest further ice growth. AFPs have gained a large interest for their use in antifreeze formulations for water-based materials, such as foods, waterborne paints, and organ transplants. Instead of commonly used colligative antifreezes such as salts and alcohols, the advantage of using AFPs as an additive is that they do not alter the physicochemical properties of the water-based material. Here, we report the first comprehensive evaluation of thermal hysteresis (TH) and ice recrystallization inhibition (IRI) activity of all major classes of AFPs using cryoscopy, sonocrystallization, and recrystallization assays. The results show that TH activities determined by cryoscopy and sonocrystallization differ markedly, and that TH and IRI activities are not correlated. The absence of a distinct correlation in antifreeze activity points to a mechanistic difference in ice growth inhibition by the different classes of AFPs: blocking fast ice growth requires rapid nonbasal plane adsorption, whereas basal plane adsorption is only relevant at long annealing times and at small undercooling. These findings clearly demonstrate that biomimetic analogs of antifreeze (glyco)proteins should be tailored to the specific requirements of the targeted application.

Significance

Controlling ice crystal growth is a grand scientific challenge with major technological ramifications. Some cold-adapted organisms such as fish and arthropods are protected against ice growth by producing antifreeze proteins (AFPs). These AFPs adsorb onto embryonic ice crystals, thereby inhibiting their growth. On a macroscopic level, this is evidenced by ice recrystallization inhibition (IRI) and thermal hysteresis (TH) activity. Our research demonstrates the absence of a clear correlation between TH and IRI activities, and underlines the importance of the extent of supercooling and annealing time on the efficacy of AFPs in blocking ice growth. We emphasize that successful application of AFPs and synthetic analogues in innovative strategies for cryopreservation, cryoprotection, and antiicing technologies requires optimization tailored toward the specific purpose.


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melting temperature of the sample (20–22). Recrystallization of ice involves ice grain boundary migration (20), in which large ice crystals increase in size and small crystals disappear (i.e., Ostwald ripening). AFPs inhibit grain boundary migration processes by stopping ice from growing and melting at the boundaries (23, 24). IRI is already significant at submicromolar AFP concentrations, which is well below concentrations required for TH (25). As a result of IRI, ice crystals remain small in frozen solutions, which is essential for survival in the cold and the preservation of frozen foods and other frozen hydrated materials (26).

Despite great progress in understanding TH and IRI activity of AFPs, the relation between these activities is still highly debated (27–30). Nonetheless, visual examination of crystals in the presence of AFPs (31, 32) as well as ice etching (13) showed that direct ice binding plays a crucial role in both types of activity. Further exploitation of ice crystal growth modulation by macromolecular antifreezes in industrial applications may lead to the development of inexpensive synthetic materials that can enhance or outperform their natural counterparts (33). However, rational design of such materials can only be achieved if the underlying antifreeze function of AFPs is completely understood. It is essential to understand if and how IRI and TH activities are related to each other and the differential affinity for their various ice crystal planes.

We investigated all major classes of AFPs using nanoliter cryoscopy, sonocrystallization, and IRI activity assays. Nanoliter cryoscopy is the most commonly used method to measure the TH activity of AFPs (17). In cryoscopy, a nanoliter aqueous sample droplet is directly observed under a microscope (Fig. S1). A sample solution is first flash-frozen and then melted back until a small single ice crystal is obtained. Samples are then further cooled at a very slow rate, until a sudden burst of ice growth is observed. The TH determined in this manner is defined as the difference between the melting point and the nonequilibrium freezing point, which is the temperature at which ice starts to grow in a sudden burst. The major advantage of nanoliter cryoscopocy is that a single measurement requires only several nanoliters of AFP solution. However, factors such as cooling and melting rate, annealing time, and initial crystal size affect the determination of the TH activity (34). To overcome these limitations, Gaede-Koehler et al. developed a highly accurate, time-independent method based on sonocrystallization to determine
TH activity (Fig. S2) (35). In sonocrystallization, a sample solution is slowly supercooled by approximately −6 °C, followed by a short ultrasound pulse resulting in nucleation and freezing. Slow melting of the sample allows for the determination of the freezing and melting points within a single experiment. The sonocrystallization method provides control of the degree of supercooling and rate of freezing, excludes any observer-based bias, and is independent of initial ice crystal size. IRI activity is determined quantitatively as the threshold concentration below which ice crystal nucleation method provides control of the degree of supercooling and melting points within a single experiment. The sonocrystallization method provides control of the degree of supercooling and rate of freezing, excludes any observer-based bias, and is independent of initial ice crystal size. IRI activity is determined quantitatively as the threshold concentration below which ice crystal growth is apparent in a thin wafer of sample solution in consecutive optical microscopy images taken at regular time intervals (Fig. S3).

Our results show a significant difference in TH activity measured by nanoliter cryoscopy and sonocrystallization. TH activity measured by cryoscopy is the highest for AFPs that bind to basal (and other) planes, whereas nonbasal plane binding is essential to demonstrate hysteresis using sonocrystallization. This apparent contradiction is due to the longer exposure time of nascent ice crystals to AFPs at a lower ice volume fraction in cryoscopy compared with sonocrystallization. The adsorption kinetics of AFPs also impacts TH activity and is known to depend on both AFP type and the ice plane of adsorption (36, 37). Furthermore, no significant correlation is found between TH and IRI activities. These insights provide a deeper understanding of how AFPs arrest ice crystal growth, and emphasize that successful application of antifreeze (glyco)proteins and synthetic analogs in processes and technologies relies on an optimization tailored to the targeted application.

### Results

#### Freezing Point Determination Using the Sonocrystallization Method.

Fig. 2 shows a typical sonocrystallization measurement of an aqueous solution of the recombinantly expressed quaternary-amino-ethyl (QAE)-binding isoform of AFP-III from ocean pout (rQAE). After initiation of ice nucleation by a short ultrasound pulse, latent heat is released, followed by the development of a stable freezing plateau. Slow melting of the sample allows determination of the melting point, and thus the TH gap (Fig. 2B), with high reproducibility (Fig. S2). The TH activity of recombinant rQAE and AFP-III from a natural source (opAFP-III) is proportional to the square root of the molar concentration (Fig. 2C), which is in accordance with cryoscopy data (38). The rQAE is slightly more active than opAFP-III due to the presence of inactive isoforms in the purified extract of the latter. We further determined the activity of rQAE mutants, proving that single mutations within the ice-binding site of the protein can have a marked impact on the TH activity, which underlines the delicate balance between the structure and activity of AFPs (Fig. S4).

#### Activity Ranking Based on Cryoscopy and Sonocrystallization Experiments.

Table 1 shows the TH activities of six different AFPs obtained by nanoliter cryoscopy and sonocrystallization. The six AFPs vary significantly in terms of structure, putative ice-binding plane, and TH activity. In cryoscopy, the basal plane-binding AFPs from *M. primoryensis* (MpAFP) and *Dendroides canadensis* (DAAF-1) show the highest maximum activities (TH ≤ 5–6 °C), as expected. Fish type I and III AFPs show comparable maximal activities with, for instance, TH = 0.6 °C for wAFP1 from winter flounder and rQAE at 5 mg/mL and 3 mg/mL, respectively. These values are consistent with previously reported values for these proteins (38, 47). Surprisingly, in sonocrystallization, the fish AFPs show TH ≤ 0.5 °C, whereas basal plane-binding AFPs of nonfish exhibit TH ≤ 0.2 °C (Fig. S5).

To directly compare the values for the TH activity from the two methods, we have determined the ratio \( TH / \sqrt{C} \) corresponding to the slope of the linear relation between TH and the square root of the protein concentration as shown in Fig. 2C and Fig. S6.

![Fig. 2.](image)

(35) Olijve et al.
This reflects the efficiency of the AFPs rather than their efficacy (i.e., maximal activity). Ordering AFPs based on cryoscopy from the most to the least active gives MAPFp (20.8) > DAFP-1 (19.8) > rQAE (0.90) > AFPgp (0.78) > ssAFP1 (0.57) > wFAFP1 (0.48). The ranking based on sonocrystallization is entirely different: rQAE (0.91) > MAPFp (0.85) > AFPgp (0.48) > DAFP-1 (0.32) > ssAFP1 (0.13) > wFAFP1 (0.07). Remarkably, rQAE is the most active AFP in the sonocrystallization assay with THsono/√C = THnano/√C. MAPFp is 25 times less active in sonocrystallization and shows only a small TH gap in sonocrystallization (Fig. S5C). Also, DAFP-1 (0.32) displays a dramatic 62-fold decrease in activity. The small α-helical proteins ssAFP1 (0.13) and wFAFP1 (0.07) show only minimal activity in a sonocrystallization assay (Fig. S5 E and F).

Out of all investigated AFPs, only AFP-III and AFPgp show a comparable TH activity in cryoscopy and sonocrystallization. Interestingly, ice etching revealed that both these proteins bind predominantly to the six equivalent {1010} primary prism planes (Fig. 1 and Table 1). Ice etching studies on type I AFPs demonstrated binding of wFAFP1 to the 12 equivalent {2021} pyramidal planes and ssAFP1 to the six equivalent {1120} secondary prism planes. Most striking is the poor performance of the insect and bacterial AFPs in sonocrystallization, even though these proteins have been reported to bind both prism and basal planes. This dual recognition is thought to be the basis for their high activity in cryoscopy (18, 48).

**Correlation Between IRI and TH Activities.** All investigated AFPs with confirmed TH activity show IRI activity at low (micromolar) concentrations, from which we have determined the IRI efficacy (C), which represents the effective AFP concentration below which no IRI activity is observed (Table 1 and Fig. S7). The measured C for rQAE = 5.9 μM, wFAFP1 = 5.8 μM, and AFPgp = 0.00091 μM in this work are similar to values reported by Budke et al.: natural AFP-III = 0.49 μM, natural AFP-I variant S35 = 6.1 μM, and natural AFPgp = 0.001 μM (22). The difference between C for rQAE in our study and natural AFP-III in the study of Budke et al. may be ascribed to a difference in protein purity of the natural extract.

Strikingly, the IRI experiments yield the highest IRI activity for AFPgp, whereas only moderate TH activity is observed for AFPgp in both cryoscopy and sonocrystallization. The hyperactive DAFP-1 with 20- to 40-fold higher THnano/√C activities than rQAE and wFAFP1 shows only moderate IRI activity. This contradictory observation has also been noted by other groups (27, 28). We have calculated the Pearson correlation coefficient (ρ) of the TH and IRI activity parameters determined from the sonocrystallization (THnano/√C), nanoliter cryoscopy (THnano/√C), and IRI (C) experiments, but found no significant correlation (Table S1).

**Discussion**

In this study, we report nanoliter cryoscopy, sonocrystallization, and ice recrystallization inhibition experiments on all major classes of AFPs to investigate the factors that govern both TH and IRI activity. Using TH/√C as a quantitative measure for TH activity, we have ranked AFPs by sonocrystallization to rQAE > MAPFp > AFPgp > DAFP-1 > ssAFP1 > wFAFP1, which differs significantly from the ranking based on cryoscopy, in which MAPFp and DAFP-1 are by far the most active. It seems that all AFPs that bind prismatic planes (rQAE, AFPgp, MAPFp, and DAFP-1) give a significant noncolligative freezing point depression in sonocrystallization; these are ranked one through four out of six. AFPs that solely target the secondary prism (ssAFP1) or pyramidal planes (wFAFP1) show, on molar basis, very little activity. Surprisingly, “hyperactive” AFPs perform poorly in sonocrystallization, with a 25-fold (MAPFp) and 62-fold (DAFP-1) lower THnano/√C, which is even lower than the activity of the “moderate” rQAE. Adsorption of hyperactive AFPs onto both prism and basal planes has been suggested to enhance their ability to depress the nonequilibrium freezing point, making them more potent than their moderately active counterparts that target a single, nonbasal ice crystal plane (18). Our findings clearly demonstrate that primary prism plane binding is a far more important determinant of THnano/√C than basal plane binding.

This raises the question: why are hyperactive AFPs potent in cryoscopy yet inefficient in sonocrystallization? We propose that the binding of the hyperactive AFPs to basal planes is less relevant in the sonocrystallization experiments, because blocking ice growth along the fast ice growth a-axis direction is more important. The initiation of ice crystallization in a sonocrystallization assay results from many nucleation sites, and ice growth is very fast because the sample is significantly undercooled. The most favorable and fastest growing ice lattice direction is along the a-axis direction {1120} (13, 49). Therefore, binding to prism planes is crucial, as only adsorption onto this plane blocks the predominant growth mode. Ice grows fastest along the a-axis direction, because here, chains or networks of water molecules can cooperatively hydrogen bond to the ice lattice, having one hydrogen bond per water molecule to attach to the ice surface. The frontal views of the specific ice crystal planes depicted in Fig. 1 illustrate how the extensive hydrogen-bonding network of the water molecules in the ice lattice favors the attachment of new water molecules. Ice grows fast, but less fast, along the {1010} direction because only pairs of water molecules can attach here, having one hydrogen bond per two water molecules interact with the ice surface. Ice growth along the c axis requires 2D nucleation on the molecularly smooth basal plane, which makes ice growth there slow. HypAFP adsorption onto the slowly growing basal planes is thus irrelevant at moderate undercooling when rapid ice growth proceeds mainly by attachment at the prism planes.

**Table 1. TH and IRI activity of AFPs from nanoliter cryoscopy, sonocrystallization, and optical microscopy measurements**

<table>
<thead>
<tr>
<th>AFP</th>
<th>Ice crystal-binding plane</th>
<th>THnano/√C, °C cmM−1/2</th>
<th>THnano/√C, °C cmM−1/2</th>
<th>CI, μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>rQAE</td>
<td>{1010} primary prism and {2021} pyramidal planes</td>
<td>0.90</td>
<td>0.91</td>
<td>5.9</td>
</tr>
<tr>
<td>AFPgp</td>
<td>{1010} primary prism planes (40, 41)</td>
<td>0.78</td>
<td>0.48</td>
<td>0.00091</td>
</tr>
<tr>
<td>MAPFp</td>
<td>prism and basal planes (42)</td>
<td>0.62</td>
<td>0.85</td>
<td>0.011</td>
</tr>
<tr>
<td>DAFP-1</td>
<td>prism and basal planes (43)</td>
<td>19.8</td>
<td>0.32</td>
<td>2.1</td>
</tr>
<tr>
<td>ssAFP1</td>
<td>{1120} secondary prism planes (13)</td>
<td>0.57</td>
<td>0.13</td>
<td>n.d.</td>
</tr>
<tr>
<td>wFAFP1</td>
<td>{2021} pyramidal planes (13)</td>
<td>0.48</td>
<td>0.07</td>
<td>5.8</td>
</tr>
</tbody>
</table>

The ratio TH/√C is used as a quantitative measure of TH activity. It corresponds to the slope of the curve that describes TH as a function of the square root of the protein concentration as illustrated in Fig. 2C. CI is the IRI efficacy and represents the inhibitor concentration determined from the inflection point of a curve of the recrystallization constant k vs. C. (21). The tabulated cryoscopy values are from this work for rQAE, AFPgp, and DAFP-1 and from literature for wFAFP1 (44), MAPFp (45), and ssAFP1 (46). Boldfaced show the two AFPs with the highest measured activity for a particular activity assay.
Fluorescent microscopy studies showed that hypAFPs adsorb to both prism and basal planes; however, little is known about the difference in surface coverage (50). A complete arrest of ice growth requires a sufficiently high surface coverage, which is attainable if the rate of AFP adsorption (which is directly related to the AFP concentration in solution) is fast compared with the growth rate of the ice crystal plane (37). If the AFP concentration and/or the AFP adsorption rate are too low, ice-bound proteins will get engulfed. A recent fluorescence study by Drori et al. reports similar adsorption rates for rQAE and TmAFP (a structural analog of DAFP-1) at the prism plane (36); however, TmAFP adsorption at the basal plane was much slower. Relatively slow hypAFP adsorption at the basal planes only becomes relevant during prolonged annealing at minor supercooling and thus modest ice growth rates. The high values for TH in nanoliter cryoscopy assays arise due to long exposure times at slow cooling rates and modest supercooling, which accommodates slow hypAFP adsorption onto basal planes. By contrast, ice growth is very rapid during a sonocrystallization experiment at several degrees of supercooling, which warrants fast and significant AFP accumulation at the prismatic planes to arrest ice growth. Apparently, rQAE accumulates with a high adsorption rate at sufficient coverage on the primary prism planes to block rapid growth in sonocrystallization experiments, giving rise to a similar $TH_{\text{sonos}} / \sqrt{C}$ to $TH_{\text{unson}} / \sqrt{C}$, which is unique for rQAE.

A unified theory to describe how structurally different AFPs accommodate TH activity thus needs to take the following factors into account: (i) ice-binding plane of AFP, (ii) ice adsorption rate of the AFP, (iii) surface coverage, (iv) degree of undercooling or cooling rate (and thus speed of ice growth), and (v) kinetics and mechanism of ice nucleation (51, 52). Because all these factors are different in sonocrystallization and cryoscopy for the various AFPS, no significant correlation is observed. Furthermore, the degree of undercooling can significantly change the antifreeze properties of the AFP. Feeney and coworkers reported that AFGP9 shows only a freezing point depression when freezing is initiated after modest supercooling ($\text{--1}^\circ \text{C}$), which is absent when frozen after deep supercooling ($\text{--6}^\circ \text{C}$) (53, 54). The loss of AFGP9 activity was hypothesized to be related to differences in the structure of the ice crystal planes. If the ice surface is “rough” due to imperfections in the ice crystal planes, as in the case of ice grown at $\text{--6}^\circ \text{C}$, then the energy requirement for ice growth is low. If the ice surface is smooth, ice growth depends on surface nucleation to form a new ice crystal layer. Unraveling the relative importance of these factors warrants detailed physicochemical experiments (single-molecule imaging, force spectroscopy, sum frequency generation spectroscopy on the ice/water interface, etc.) and accurate (molecular dynamics) simulations (55–57).

In conclusion, the TH and IRI activity of AFPS as determined by cryoscopy, sonocrystallization, and optical microscopy are not correlated due to differences in ice adsorption behavior and operating conditions. These different activities highlight the strength and weakness of the different AFPS related to their biological role. For example, the hypAFPs produced by insects need to block ice growth when temperatures drop far below 0 °C (i.e., large TH under slow cooling conditions). On the other hand, fish living in polar sea water with only modest temperature variations require rapid ice blocking and very effective IRI activity, as the AFP-stabilized ice crystals remain in their body fluids throughout their lifespan because the endogenous ice crystals do not melt at warmer temperatures in summer (58). These findings demonstrate that the application of AFPS and synthetic analogs requires a tailored optimization to the specific purpose. Especially, rational design strategies for synthetic ice binders should not focus on compatibility with all ice crystal planes, but, instead, factors such as cooling rate, extent of undercooling, annealing times, etc., of the intended application need to be taken into account. Furthermore, we propose abandonment of the categorization of AFPS into moderate and hyperactive variants, as this is based on an activity ranking that is method- and protocol-dependent. These new insights provide a deeper understanding of the underlying mechanism that governs IRI and TH activity, which supports the development of synthetic macromolecular antifreezes for cryoprotection, cryopreservation, deicing, and anticicing technologies.

Materials and Methods

Protein Samples. Recombinant expression and purification of rQAE (55), DAFP-1 (59), and MpAFP (42) was performed as described previously. The MpAFP was synthesized using solid-phase peptide synthesis as described previously (60). The ssAFP1 and AFGP1-5 were purified from short-horned sculpin and Antarctic tooth fish blood serum by gel filtration chromatography. The ssAFP-III was purchased from A&F Protein Inc. and used without further purification. All other chemicals were purchased from Sigma Aldrich and used as received.

TH and IRI Experiments. The Clifton nanoliter cryoscopy (44), sonocrystallization (55), and IRI (21, 22) experiments were performed as described elsewhere. All samples were measured in 20 mM Tris, pH 7.5 buffer, except MPAFP, which was measured in 20 mM Tris, 2 mM CaCl2, pH 7.5 buffer.

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Supporting Information

Olijve et al. 10.1073/pnas.1524109113

Fig. S1. Illustration of the typical procedure of the nanoliter cryoscopy measurements. (a) A nanoliter sample droplet immersed in an oil droplet is rapidly cooled to $-40^\circ$C to form polycrystalline ice. (b) The sample is slowly melted to form a single ice crystal (c) which is held just below the melting point of the sample. (d) The sample is slowly cooled until a sudden ice crystal burst is observed. The TH is defined as the difference between the melting point of the solution and the temperature at which the ice crystal burst is observed. A single ice crystal shown in the micrographs is cooled from $-0.2^\circ$C (I) to $-0.3^\circ$C (IV) at a rate of 0.05 °C/min, in a solution of fish type III AFP from Notched-fin eelpout (0.04 mM). Micrographs are adapted from ref. 61.

Fig. S2. (A) In a typical sonocrystallization, a 1-mL sample is slowly cooled until $\sim -6^\circ$C, after which a short sonopulse is applied to initiate freezing. The sonocrystallization measurements demonstrate a high reproducibility over the entire temperature profile, as shown by three repeat measurements of rQAE at a concentration of 1 mg/mL in 20 mM Tris, pH 7.5. The temperature readout accuracy of the experimental setup is within 0.01 °C. (B) Measurements on the AFP samples have high reproducibility with a deviation in determining the nonequilibrium freezing temperature below $< 0.02^\circ$C.
Fig. S3. Illustration of the typical procedure for the IRI experiments. (a) A sample droplet (1 μL) of the analyte dissolved in 30 wt/wt% sucrose solution is rapidly cooled to −40 °C at a rate of 20 °C/min, (b) heated to −7 °C at a rate of 10 °C/min, and (c) held at constant temperature for 120 min. Micrographs are taken at regular time intervals, and the rate of recrystallization is determined using automated image analysis software.

Fig. S4. (A) Identified ice-binding face of type III AFP (QAE isoform), showing the central position of the Thr18 residue at the ice-binding face that recognizes the primary prism plane. Image reprinted from ref. 62. (B) Neutron crystal structure (PDB: 3QF6) shows the specific interaction of Thr18 with a tetrahedral water cluster. Image reprinted from ref. 63. (C) Sonocrystallization measurements of wild type rQAE and mutant rQAE with a single mutation on the Thr18 position. The T18D mutant shows a significant reduction in activity, whereas T18N has no freezing hysteresis activity at all. This shows that the adsorption of rQAE to the pyramidal or primary prism planes can be disrupted by a single mutation.
Fig. S5. Sonocrystallization data of (A) rQAE, (B) AFGP_{1-5}, (C) MpAFP, (D) DAFP-1, (E) ssAFP1, and (F) wFAFP1. The freezing hysteresis gap is indicated by arrows. All proteins were measured in 20 mM Tris buffer, pH 7.5, except MpAFP, which was measured in 2 mM CaCl\(_2\) 20 mM Tris buffer, pH 7.5.
Fig. S6. Concentration-dependent TH activity of (A) rQAE, (B) AFGP\textsubscript{1–5}, (C) MpAFP, (D) DAFP-1, (E) ssAFP1, and (F) wfAFP1 from sonocrystallization and nanoliter cryoscopy. Dashed line illustrates a linear fit of the data points, of which the slope gives the ratio $\text{TH/C}^{1/2}$ and is used as a measure to directly compare AFP activity of sonocrystallization with cryoscopy. An average molecular mass ($M_r = 15$ kDa) was assumed to compute the molar concentration of AFGP\textsubscript{1–5}. 

Olijve et al. www.pnas.org/cgi/content/short/1524109113
Fig. S7. Quantitative IRI measurements of (A) rQAE, (B) AFGP, (C) MpAFP, (D) DAFP-1, and (E) w/ AFP1. The recrystallization constant $k_d$ is determined as a function of AFP concentration. $C_i$ represents the IRI activity and is determined from the inflection point of the s curve.
Table S1. Pearson correlation coefficient ($\rho$) of the TH and IRI activity parameters determined from the sonocrystallization (sono, $TH_{nano}/\sqrt{C}$), nanoliter cryoscopy (cryo, $TH_{sono}/\sqrt{C}$), and IRI (I) experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$\rho$</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sono – cryo</td>
<td>0.30</td>
<td>0.57</td>
</tr>
<tr>
<td>Sono – IRI</td>
<td>−0.19</td>
<td>0.76</td>
</tr>
<tr>
<td>Cryo – IRI</td>
<td>−0.54</td>
<td>0.35</td>
</tr>
</tbody>
</table>

A perfect positive correlation between the activity parameters would be denoted by $\rho = +1$, and a perfect negative correlation by $\rho = −1$. Values of $−0.5 < \rho < 0.5$ represent no significant or weak correlation. $P$ values $> 0.05$ represent no statistical significance.