

Intrinsically Disordered Regions in CAHS Proteins Govern Self-assembly and Enzyme Stabilization Against Multiple Stresses

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Abstract

Cytosolic Abundant Heat Soluble (CAHS) proteins are produced by extremotolerant tardigrades as a protective mechanism to stabilize cellular components, such as enzymes, in response to environmental stress. However, the molecular principles governing their protective function have only begun to be elucidated. Herein, we analyze nine CAHS proteins containing intrinsically disordered regions (IDRs) of different lengths to investigate their key role in self-assembly and enzyme stabilization. We show that CAHS proteins with a longer N-terminal IDR present a higher propensity for self-assembly at nano- and macro-scale. The propensity for self-assembly also correlates with their ability to stabilize enzymes, with CAHS proteins containing longer IDRs exhibiting up to 10-fold increase in enzyme stability when exposed to desiccation stress. Beyond desiccation, we reveal new stabilizing roles of CAHS assemblies under a broad variety of abiotic stresses, including extreme pH conditions, high temperatures, addition of organic co-solvents, long-term storage, and long operational times. This concept is further demonstrated in a bi-enzymatic cascade reaction composed of an ADH (Alcohol dehydrogenase) and a NOX (NADH oxidase) for the synthesis of a valuable natural product (peryllaldehyde). The addition of CAHS proteins resulted in a 3-fold increase in product formation compared to the bi-enzymatic cascade not supplemented with these proteins. Our findings not only reveal new structural insights into the functional roles of CAHS proteins but also introduce a new bioinspired platform to overcome the challenges of enzyme stability for biotechnological applications.

Introduction

Enzymes play essential roles in biocatalysis due to their remarkable selectivity, biocompatibility, and biodegradability, with an expanding range of applications in biotechnology, biosensing, and biomedicine^{1,2}. Yet, enzymes exhibit limited stability under the harsh conditions often required for biotechnological and industrial processes, such as high temperature, presence of organic cosolvents, or extreme pH values. This dramatically impacts the efficiency and sustainability of biocatalytic reactions. To address this challenge, various strategies have been developed with partial success³. Enzyme discovery efforts have focused on extremophilic organisms as a source to find more robust enzymes⁴. However, enzymes that are stable under one type of harsh conditions (e.g., high temperature) may not be stable when exposed to other harsh conditions (e.g., low pH). Despite many advances in bioinformatic tools and protein engineering in the last few years, enzyme stability remains a major challenge^{5,6}. Enzymes require a certain structural flexibility to perform the biocatalytic reaction, but excessive mobility increases their vulnerability to denaturation. Enhancing enzyme stability is therefore a key objective for expanding the applicability of biocatalysts across industrial and biotechnological processes^{7,8}.

In nature, tardigrades (also known as water bears) have developed an alternative strategy to protect their enzymes under extreme conditions. Recent studies have shown that these microscopic animals produce Intrinsically Disordered Proteins (IDPs) that can protect not only enzymes but also other biological structures, such as membranes, under stress conditions⁹. These IDPs have been designated as

Tardigrade Disordered Proteins (TDPs) as they present a unique sequence conservation to tardigrades and do not resemble other IDPs.

Among the three families of TDPs, Cytoplasmic Abundant Heat Soluble (CAHS) proteins are found in the cytoplasm of tardigrade cells and have been shown to protect enzymes when exposed to desiccation conditions (Fig. 1a)¹⁰. These proteins were only discovered in 2012, thus, their mode of action for protein stabilization *in vivo* and also *in vitro* remains to be completely understood^{11,12}. In recent years, several CAHS proteins have been identified across tardigrade genomes, however, we still lack a comprehensive characterization of the functionalities of this protein family. The majority of investigations have been strongly focused on CAHS D, a TDP from the tardigrade *Hypsibius exemplaris*^{10,13,14}. In addition to this, tolerance to extreme conditions varies among tardigrade species. For instance, *Paramacrobiotus richtersi* and *Ramazzottius varieornatus* can withstand much higher levels of desiccation compared to *Hypsibius exemplaris*.

CAHS proteins present a central α -helix domain flanked by two intrinsically disordered regions (IDRs) (Fig. 1b). The helical domain, also known as the linker, has been recently reported to play a crucial role in the stabilizing properties of CAHS proteins^{15–18}. Moreover, the central α -helix has been related to the ability of CAHS proteins to self-assemble into gel-like structures which are relevant for their biological function^{10,19}. In contrast, the terminal intrinsically disordered regions (IDRs), although implicated in the self-assembly of CAHS proteins, have received much less attention to date and their functional role remains largely unexplored.

Among the various environmental harsh conditions, desiccation is the most widely studied condition for demonstrating the protective properties of CAHS proteins^{13,20}. This focus is due to the remarkable ability of tardigrades to undergo near-complete desiccation in response to external harsh conditions, which is known as cryptobiosis. During this process, tardigrades transition into a specialized dormant state, where CAHS proteins play a critical role in preserving cellular integrity¹⁰. Nevertheless, tardigrades exhibit remarkable tolerance to a wide range of harsh conditions, such as high temperatures, freezing, osmotic shock, or even the vacuum pressure in outer space²¹. Many of these conditions are likewise relevant to enzyme stability. Therefore, enzyme stabilization by CAHS proteins under these conditions would serve as a valuable tool for many biotechnological applications of enzymes but has not been explored yet.

In this work, we aim to elucidate how the structural diversity of CAHS proteins influences their biological functions and how this could be a potential biotechnological tool to boost enzymatic reactions. By integrating bioinformatic analysis and systematic biochemical characterization of CAHS proteins, we aim to better understand the role of the IDRs in self-assembly and, consequently, in their stabilization properties. Beyond the state of the art, we show that different CAHS proteins protect enzymes not only against desiccation conditions but also against a variety of harsh conditions (high temperature, addition of organic cosolvents, and extreme pH). As a proof of concept, we investigate the stabilizing effects on the model enzyme NADH oxidase from *Lactobacillus pentosus* (NOX), which is poorly stable even at

storage conditions (4°C)²². Finally, and given the high relevance of NOX as a cofactor recycling system in multi-enzyme cascades^{23–25}, we demonstrate how CAHS-mediated enhancement of NOX directly improves the overall performance of a bi-enzymatic cascade for the synthesis of a small-molecule natural product.

Results

Sequence and structure diversity of CAHS proteins

We analyzed a set of nine CAHS proteins from three tardigrade species (*H. exemplaris*, *P.richtersi*, and *R. varieornatus*) with different tolerance to desiccation (Fig. 1c). CAHS1, CAHS2, CAHS5, CAHS7, and CAHS8 have been previously expressed in *E. coli* and tested for the protection of a model enzyme (see Table S1 for details in the nomenclature)^{9,26,27}. Based on those protein sequences, we next applied homology-based mining and identified four new uncharacterized CAHS proteins, named CAHS3, CAHS4, CAHS6, and CAHS9. CAHS4 showed > 99% homology with the well-known CAHS D, serving as our basis for comparison with previous studies¹⁰. Unexpectedly, sequence analysis of the nine CAHS proteins revealed low sequence homology among the CAHS proteins, with values as low as 24% (Fig. 1d). The phylogenetic tree derived from the protein homology analysis did not display a closer relationship between the CAHS proteins originating from the same tardigrade species (e.g., CAHS1-2; CAHS3-5; CAHS6-9) (Fig. S1a).

To identify the structural motifs of our set of CAHS proteins, a residue conservation analysis was performed, showing a region on the order of 100 residues that is conserved among all CAHS proteins (Fig. 1e and S1b). Further computational predictions using AlphaFold 3, Metapredict V3, and IUPred3 indicated that the conserved region resides within the most structurally constrained domain, the central α -helix linker (Fig. S2). This structured region was confirmed experimentally by circular dichroism (CD), in agreement with previous structural analysis of CAHS proteins (Fig. S3a-b)^{15,18,19}. While the size of the central α -helix linker was similar for all CAHS proteins, the computational analysis revealed significant differences in the length of the IDR at the N-terminus of the proteins (Fig. 1c and Fig. S4). CAHS5 and CAHS6 showed a 2-fold longer IDR (262–276 residues) than most of the other CAHS proteins (86–124 residues), while CAHS9 presented an intermediate length of the IDR (176 residues). Driven by these results, we further analyzed how variations in N-terminal IDR length influence the properties of CAHS proteins.

A longer IDR promotes self-assembly of CAHS proteins

The protective properties of CAHS proteins have been related to their ability to undergo a phase transition from solution to gel-solid state^{10,28}. Thus, we asked whether the differences in the IDR length of CAHS proteins are related to their ability to self-assemble, resulting in gel formation and ultimately, influencing their protective properties. Previous studies have highlighted a strong correlation between

the tolerance to desiccation granted from synthetic IDRs/IDPs and specific sequence descriptors³¹. Sequences granting the highest desiccation protection were reported to feature higher mean homotypic interaction strength (ϵ) – i.e. more self-repulsion –, a higher fraction of alanine residues, and lower sequence complexity and hydrophobicity³¹. Analysis of our set of CAHS proteins (Fig. 2a-b and Fig. S5) revealed that they span a wide range of these descriptors, particularly in terms of mean interaction strength and alanine fraction. Notably, we found that proteins with longer N-terminal IDRs (e.g., CAHS6) also display considerably higher self-repulsion, increased alanine content and lower sequence complexity. In order to better elucidate this observation, we selected three representative CAHS proteins i) spanning most of the observed descriptor space (Fig. 2a-b), ii) comprising short (CAHS7), medium (CAHS9), and long (CAHS6) N-terminal IDRs, and iii) originating from the same tardigrade species, enabling direct comparisons. Both intra-chain contact maps and inter-chain interaction matrices, generated using STARLING³² and FINCHES³³ respectively, confirmed that the three selected CAHS proteins display markedly different behavior, ranging from attractive (CAHS7, shortest N-terminal IDR) to moderately (CAHS9) and strongly repulsive (CAHS6, longest N-terminal IDR), and making them prime candidates for experimental characterization.

These proteins were recombinantly expressed in *E. coli* and purified by heat lysis obtaining a high purity grade (> 95%) (Fig. S3c). Following a previously reported methodology¹⁰, we examined the ability of these three purified CAHS proteins to form macroscopic gels as a function of protein concentration^{10,29}. In agreement with previous studies, all three proteins remained in solution at concentrations below 5 mg mL⁻¹ (Fig. 3a). Gel formation was induced by increasing the concentration of CAHS proteins, with a transition observed at 10 mg mL⁻¹, but no significant distinctions among the three CAHS proteins were detected at the macroscale.

Computational analysis using Aggrescan4D and IUPred3^{34,35}, however, revealed a correlation between IDR length and both aggregation propensity and disorder, respectively. CAHS7 (short IDR) exhibited the lowest propensity, whereas CAHS6 (long IDR) displayed the highest (Fig. 3b). Moreover, positive Aggrescan4D scores were found almost exclusively in the IDRs of all the CAHS proteins analyzed herein, suggesting that these regions may play a crucial role in the self-assembly triggering gel formation (Fig. S6). We then proposed that variations in IDR length could drive distinct self-assembly behaviors in CAHS proteins at smaller scale (micro- or nanoassemblies). To test this hypothesis, we first conducted turbidity measurements (Abs 595 nm) of CAHS7, CAHS9, and CAHS6 at protein concentrations below the clear gel formation (< 5 mg mL⁻¹). In all cases, the turbidity increased as a function of protein concentration, suggesting a concentration-dependent self-assembly even at low protein concentrations (Fig. 3c). Furthermore, a trend was observed between the turbidity of CAHS proteins and the length of their IDR (CAHS6 > CAHS9 > CAHS7). CAHS6 exhibited an exponential gain in turbidity, reaching values 5-fold higher than those observed for CAHS7. Clear differences in the size distributions of the CAHS nanoassemblies could be distinguished by Dynamic Light Scattering (DLS) analysis. CAHS7 and CAHS9 showed unimodal distributions of 90 and 120 nm in diameter, respectively, revealing relatively homogeneous and smaller assemblies at the nanoscale (Fig. 3d). In contrast, CAHS6 displayed a

bimodal distribution, with two populations at 600 nm and at 60 nm. These results support the hypothesis that the formation of supramolecular nanoassemblies depends on the IDR length (Fig. 3e).

Additionally, CAHS6 self-assemblies at the microscale were analyzed by confocal fluorescence microscopy. Since previous works have indicated that IDRs may transition into β -sheet and form β -sheet stacking during self-assembly, Thioflavin T (ThT) was used as a fluorescent probe for visualization^{36,37}. ThT fluorescence intensity is a common reporter of β -sheet structures due to the increase in its quantum yield upon binding to these structures. This analysis proved the formation of CAHS6 self-assemblies also at the microscale, demonstrating that CAHS proteins can self-assemble across scales (Fig. 3f). Remarkably, micro-assemblies of CAHS6 were detected even at concentrations ($< 5 \text{ mg mL}^{-1}$) at which gel-formation was not observed macroscopically.

To further confirm these findings, Bis[sulfosuccinimidyl] suberate (BS3) was employed as a crosslinker to stabilize CAHS protein-protein interactions, enabling the visualization of CAHS nano-assemblies by SDS-PAGE (Fig. S7). This analysis confirmed that CAHS6 protein can self-assemble into higher-order structures even under more diluted conditions ($0.5\text{-}2 \text{ mg mL}^{-1}$).

CAHS protein-protein interactions during self-assembly

CAHS proteins can assemble into hydrogels that dissociate back to solution upon temperature increase, as CAHS are heat-soluble proteins^{10,38}. We therefore investigated how the distinct IDRs impact the strength of protein-protein interactions underlying assembly. Upon exposing CAHS7, CAHS9, and CAHS6 to increasing temperatures, we observed that CAHS6 (long IDR) assemblies remained stable at substantially higher temperatures (45°C) compared to those formed by CAHS9 (medium IDR) and CAHS7 (short IDR) (Fig. 4a). Moreover, the turbidity values at all tested temperatures followed the same trend as observed in our previous experiments (CAHS6 > CAHS9 > CAHS7), suggesting differences in assembly size, with CAHS6 forming the largest assemblies.

It has been previously suggested that CAHS proteins may transition into more ordered structures (i.e., β -sheets) that play a relevant role in protein assembly¹⁰. To identify differences in the IDRs, AlphaFold 3 Multimer predictions were carried out to computationally model the self-assembly of CAHS proteins³⁹. We identified a trend between the IDR length and the minimum oligomeric state presenting a structural transition from IDRs to β -sheets which indicate self-assembly. CAHS6 showed β -sheet stacking with only three monomers, while CAHS9 and CAHS7 required at least five monomeric units (Fig. 3b and S8). Consequently, IDR length appears to be a primary determinant of the nucleation threshold and assembly organization of CAHS proteins. Consistent with this hypothesis, the number of secondary structure elements (particularly β -strand regions) predicted by AlphaFold 3 in the N-terminal IDRs of the investigated CAHS proteins increases dramatically in the longest IDRs (CAHS5 and CAHS6) (Fig. S9). AlphaFold 3 predictions also showed that self-assemblies of CAHS proteins form fibril-like structures, which are driven by coiled-coil interactions and β -sheet stacking (Fig. 4b). These structures are typically

driven by clusters of hydrophobic residues (Val, Leu, and Ile) and stabilized by negatively charged residues (Asp and Glu)^{40,41}. Thus, we carried out computational analysis using CapiPy which revealed a higher density of hydrophobic and negatively charged clusters in CAHS6 compared with CAHS9 and CAHS7 (Fig. 4c-d and Table S2). This enriched cluster profile likely drives the increased propensity for β -sheet formation and, subsequently, intermolecular association observed in CAHS6⁴². Particularly, more clusters of hydrophobic residues were identified in the longer IDRs.

We then experimentally confirmed the increased β -sheet-rich protein content in the different CAHS assemblies using a ThT fluorescence assay, which detects β -sheet-rich protein structures (Fig. 4e). For all three proteins, an increase in ThT fluorescence intensity was observed upon increasing protein concentration (within 20–60 μ M). The fluorescence data manifested a clear length-dependent trend, where CAHS6 displayed the highest fluorescence intensity, followed by CAHS9 and CAHS7.

Since tardigrades produce CAHS proteins in response to desiccation, we also studied the CAHS protein-protein interactions under these conditions⁹. ThT fluorescence assays were also performed after a desiccation-rehydration cycle of CAHS proteins. The self-assembled states of CAHS7 and CAHS9 remained unaffected by desiccation (Fig. 4e-f). In contrast, desiccation promoted the formation of larger assemblies of CAHS6 at low protein concentration (20 μ M; 0.94 mg mL⁻¹) (Fig. 4f). DLS measurements confirmed that the desiccation of CAHS6 led to the emergence of an additional population of larger protein assemblies (400 nm) that was absent in non-desiccated samples at this low protein concentration (Fig. 4g). To deepen our understanding of the formation of CAHS6 self-assemblies under desiccation, we characterized the structural transitions that CAHS6 undergoes under these conditions. CD spectroscopy analysis of CAHS6 was conducted under desiccation-mimicking conditions facilitated by the addition of the co-solvent trifluoroethanol (TFE). Data showed how increasing concentrations of TFE led to a progressive gain in ordered secondary structures (specially β -sheets), accompanied by a reduction in IDRs (Fig. 4h and Fig. S9). These results indicate the transition of IDR towards more ordered structures and the subsequent protein-protein interactions that trigger self-assembly.

A longer IDR in CAHS proteins improves enzyme stability against desiccation

Despite the desiccation protection of CAHS proteins has been previously demonstrated^{9,43}, the role of the IDR length in this protection has not been investigated. Therefore, using CAHS7 (short IDR), CAHS9 (medium IDR), and CAHS6 (long IDR) as our representative proteins, the stability of NOX was tested after a cycle of desiccation-rehydration. In agreement with previous studies, we observed that higher CAHS protein concentrations led to a higher stability of NOX after desiccation (Fig. 5a and S10). Importantly, we identified a direct relation between the IDR length and the stabilizing effect of the CAHS proteins. At 40 μ M, CAHS7 maintained only 36% of NOX activity, while CAHS6 retained 82% (Fig. 5b). To evaluate the broader applicability of this protective mechanism, we assessed these CAHS proteins for the

stabilization after desiccation-rehydration of another enzyme, an alcohol dehydrogenase (ADH) from *Geobacillus stearothermophilus*. Similar results were obtained with CAHS7 and CAHS6 retaining 53% and 86% of enzymatic activity, respectively, confirming the crucial role of the N-term IDR length and highlighting CAHS proteins as a versatile platform for enzyme stabilization. (Fig. 5b and S11).

Additionally, we performed the desiccation stability test with all the other CAHS proteins we had selected for this work. All CAHS proteins with a short IDR (CAHS1, CAHS2, CAHS3, CAHS4, CAHS7, and CAHS8) showed a similar stabilizing profile, retaining 45% of NOX activity after desiccation, while proteins with a long IDR (CAHS5 and CAHS6) showed a 2-fold higher NOX stability (> 85%) compared to all other CAHS proteins (Fig. S10 and Fig. S11). Notably, we did not observe any significant variation between CAHS proteins originating from tardigrade species with different stress tolerance. Conversely, we observed a strong correlation between the extent of desiccation protection and the total number of secondary structure elements predicted within N-terminal IDRs (Fig. S12), consistently with our observations in Fig. 3b, S8 and S9. As a control, we also monitored enzyme stability after desiccation-rehydration in the presence of BSA. Although BSA showed a certain stabilization of NOX, even the stabilizing effect of a CAHS protein with a short IDR was almost 2-fold higher than that of BSA (Fig. S13).

Based on our previous findings identifying CAHS6 as the best candidate to form self-assemblies, we used this protein to confirm the interactions between CAHS proteins and the enzyme. After labelling the enzyme (NOX) with a fluorophore (rhodamine B) and adding ThT to visualize the CAHS6 assemblies, confocal microscopy analysis corroborated that NOX co-localized with the CAHS6 assemblies, possibly creating a protective microenvironment for the enzyme (Fig. 5c).

CAHS proteins stabilize enzymes against various abiotic stresses: pH, organic co-solvents, and temperature

Beyond tolerance to desiccation, tardigrades are also resistant to other types of abiotic stresses. However, the capacity of CAHS proteins to protect enzymes against those harsh conditions other than desiccation remains largely unexplored. In this work, we tested the stabilizing properties of CAHS proteins under typical harsh conditions that cause stress to enzymes but are often needed in biocatalytic reactions (i.e., extreme pH, high temperature, and addition of organic co-solvents). To identify differences in the protection offered by these proteins, we selected a CAHS with a short IDR and a CAHS with a long IDR to assess NOX stability after exposure to those abiotic stresses.

First, we exposed NOX to a range of pH conditions (pH 4.0–9.0) for 20 h. It is worth mentioning that the optimal pH for NOX activity lies between pH 6.0 and 6.5⁴⁴. Enzymatic activity assays showed that NOX lost nearly half of the initial activity following incubation at the optimal pH, due to the intrinsic low stability of this enzyme (Fig. 6a). Nevertheless, the activity loss was even more dramatic (90%) at extreme pH values (pH 4.0 and pH 9.0), due to protein unfolding and aggregation of NOX (Fig. S15). In contrast, samples supplemented with CAHS proteins maintained up to 5-fold higher activity. The CAHS

protein with a long IDR exhibited the strongest stabilizing effect under acidic pH. At conditions near the optimal pH (6.0-7.5), the beneficial effects of CAHS proteins were not significant (Fig. 6a).

In a subsequent experiment, NOX was incubated at 37°C, which is the optimal temperature for activity but detrimental for long-term stability, as this enzyme presents very low stability even at 4°C⁴⁴. After only 1 h of incubation at 37°C, the NOX activity of the sample supplemented with a long-IDR CAHS protein was maintained, while the CAHS protein with a shorter IDR barely conferred stabilization (Fig. 6b). Additionally, CAHS proteins can provide partial stabilization of NOX during storage at 4°C, with almost 2-fold higher activity when supplemented with the long-IDR CAHS protein (Fig. S16).

For the third stability test, NOX was exposed to different organic co-solvents (ethanol, methanol, isopropanol, acetone, acetonitrile, and DMSO). As in the previous stability tests, we observed that the CAHS protein with a longer IDR consistently stabilized NOX across all tested conditions, while the CAHS protein with a shorter IDR only presented stabilizing properties in the presence of acetone and DMSO (Fig. 6c).

The results shown in Fig. 4e demonstrated a higher level of CAHS6 self-assembly after desiccation, which may lead to higher stabilization. We therefore tested the stabilizing properties against extreme pH, high temperature, and the addition of organic co-solvents also after desiccation. Indeed, this desiccation step before exposure to harsh conditions closely mimics the natural physicochemical context in which protection mediated by CAHS proteins occurs in tardigrade cells, when these animals prepare for survival against environmental stress^{9,21}. The same experiments conducted in Fig. 6a-c were repeated after desiccation of NOX. Across all tested conditions (extreme pH, high temperature, and addition of organic co-solvents), both CAHS proteins proved to be effective in maintaining enzyme stability (Fig. 6d-f). Nevertheless, the CAHS protein with a long IDR exhibited a more pronounced protection compared to the CAHS protein with a short IDR. These results support that the protective effect of CAHS proteins is enhanced after sample desiccation, which may induce protein assembly.

These results encouraged us to also examine the application of CAHS proteins as preservatives for desiccated enzyme powder preparations, which is a typical formulation for commercial enzymes. After desiccation of NOX in the presence of CAHS6 and storage at room temperature for more than 2.5 months, 35% of the enzymatic activity was still preserved (Fig. S17a). On the contrary, storage of NOX as a protein solution retains almost no activity after a week (Fig. S17b).

CAHS proteins as a powerful tool to improve efficiency of enzyme cascades

Finally, we integrated all our findings to enhance the performance of a relevant biocatalytic cascade reaction. A two-enzyme system composed of NOX and ADH was assembled to produce perillaldehyde. This small-molecule natural product is valued for its antimicrobial, anti-inflammatory, and antioxidant properties with broad relevance to the production of flavors, fragrances, pharmaceuticals, and

agrochemicals^{45,46}. In this reaction, perillyl alcohol is oxidized to perillaldehyde by ADH, while the cofactor (NAD⁺) is continuously regenerated by NOX (Fig. 7a)⁴⁷. The biotransformations were carried out for more than 7 h under harsh conditions for these enzymes: addition of 20% of co-solvent (DMSO), which was required to enable solubility of the substrate; temperature was set at 30°C, to which both enzymes show good activity but low stability; pH was set to 7.5, which is optimal for ADH but not for NOX. Under these conditions, NOX displayed a rapid loss of activity (Fig. 7b). In contrast, the addition of CAHS proteins (either with short IDR or long IDR) mitigated this inactivation enabling the progressive increase in product formation. Specifically, the CAHS protein with a long IDR conferred the strongest enhancement, with the conversion (%) to perillaldehyde reaching more than a 3-fold improvement compared with the biocatalytic system without CAHS proteins (Fig. 7b).

Discussion

In conclusion, we have elucidated a key design-rule relationship of the N-term IDR length of CAHS proteins with broad implications for their self-assembly dynamics and protective properties. Our comparative analyses of both novel and previously reported CAHS variants evidenced the functional versatility of this protein family. Specifically, the two CAHS proteins with long IDR (CAHS5 and CAHS6) have shown higher stabilization of the NOX enzyme in comparison with all the other CAHS proteins with a shorter IDR (CAHS1, CAHS2, CAHS3, CAHS4, CAHS5, and CAHS8). This correlation has been further validated by testing an additional protein with an intermediate length (CAHS9) which showed a moderate protective effect.

Regarding protein concentration, previous studies have described the stabilizing properties of the CAHS to be concentration-dependent^{48,49} with at least 60 μ M CAHS D protein (short IDR) needed to maintain 50% of initial enzyme activity^{9,10}. In this study, we have demonstrated that a lower concentration (40 μ M) of the long IDR CAHS variant (CAHS6) resulted in an almost 100% stabilization of two different enzymes (NOX and ADH), following desiccation-rehydration (Fig. 5b). This enhanced efficiency at lower protein concentrations aligns with the higher propensity of CAHS proteins containing a long IDR to form self-assemblies. Herein, we have demonstrated that CAHS self-assembly is not restricted to macroscopic hydrogels (Fig. 3). We have identified CAHS self-assemblies in the nano- and micro-scale, under conditions that may have been overlooked due to the soluble appearance of samples. Furthermore, confocal microscopy confirmed the colocalization of CAHS proteins and the enzyme (NOX) within the micro-assemblies (Fig. 5c), suggesting a physical mechanism of entrapment or scaffolding.

Beyond the reported stabilization of a commercial enzyme against dehydration⁴³, our broad approach establishes CAHS proteins as powerful tools for the stabilization of different recombinant enzymes. For instance, the highly labile NOX enzyme exhibited an 8.5-fold increase in residual activity (from 10% to 85%) after a desiccation-rehydration cycle when supplemented with long-IDR CAHS variants (Fig. 5a). Similarly, we evaluated the ADH enzyme derived from the thermotolerant organism *Bacillus stearothermophilus*. While inherently stable at high temperatures, this enzyme remains unstable under

other harsh conditions such as desiccation (Fig. S11). Supplementation with a long-IDR CAHS protein preserved the enzyme activity, yielding almost 10-fold stability increase after rehydration. These results suggest that CAHS proteins act as versatile enzyme stabilizers, shielding enzymes from different types of stress simultaneously.

We envision these new insights into the functional relevance of the N-terminal IDR will significantly advance our understanding of the recently discovered CAHS proteins and the broader emerging field of TDPs. Ultimately, this work contributes to decoding the unique molecular mechanisms that allow tardigrades to survive nearly any stress condition. From an applied perspective, our results have untapped the potential of CAHS proteins as a simple but very effective strategy to stabilize enzymes against a wide range of harsh conditions (high temperature, non-optimal pH, presence of organic solvents, desiccation, and long operation times). The shelf-life of enzymes has also been notably enhanced by the addition of CAHS proteins, achieving more than 40% of enzyme (NOX) stability after 2.5 months of storage at room temperature, which typically maintains less than 20% activity within just 1 h (Fig. S17). Noteworthy, CAHS proteins can be easily expressed reaching outstanding recombinant protein yields exceeding 300 mg L^{-1} (Fig. S18A). This easy and efficient production, together with the quick protein purification by heat-lysis, position CAHS proteins as attractive protein stabilizing agents for biotechnological applications. Our findings open promising new research directions in the search for more sustainable and efficient methods to synthesize chemical commodities (e.g., pharmaceuticals, food additives, agrochemicals) using enzyme cascades as cell-free biocatalytic systems.

Methods

Bioinformatic Analysis

Sequences were fetched from UniProt using the IDs / Gene names provided in Table S1. Analysis for Figs. 2, S4-5 were performed in Python3 using custom scripts (analyze_tardigrade_CAHS.py, see Data Availability). Disorder prediction was performed using Metapredict v3 (<https://github.com/idptools/metapredict>) with default threshold (0.5). Sequence descriptors were computed using custom (alanine fraction) or built-in localcider (<https://github.com/Pappulab/localCIDER>) functions. Homotypic mean interaction strength (epsilon) and corresponding interaction matrices were computed using FINCHES (<https://github.com/idptools/finches>) (default window length: 31 residues), whereas intra-chain contact maps were obtained using STARLING (<https://github.com/idptools/starling/>) based on 100 conformers at 150 mM ionic strength.

For the analysis in Figure S9, structure predictions were obtained using the AlphaFold 3 Server (date: July 29th 2026). A custom Python3 script (analyze_af3_structures.py) subsets the generated structures to the N-term IDRs predicted by Metapredict v3, runs DSSP via MDAnalysis (<https://github.com/MDAnalysis/mdanalysis>) and counts the predicted secondary structure elements, divided in α -helices, β -strands and total (sum) for each of the 5 diffusion models returned by

AlphaFold 3. The script also subsets the structure from the top-ranked model to the N-term IDR and produces an optimal rendering to inspect the secondary structure elements.

Materials

All the reagents used for buffer preparation, culture media preparation, and biochemical work were purchased from Sigma Aldrich (Gillingham, UK), Thermo Fisher (Loughborough, UK), Acros Organics (Reinach, Switzerland), and Apollo Scientific and used without further purification.

Protein expression

Firstly, homology-based mining in the genome of *H. exemplaris* was applied for CAHS3 and CAHS4 based on the sequence of CAHS1. Similarly, CAHS6 sequence was identified by homology-based mining in the genome of *R. varieornatus* using CAHS5. Secondly, genes encoding the different CAHS proteins were codon optimized for expression in *E. coli* and cloned into the pET-28b vector (with a His-tag at the N-terminal) by Genewiz/Genscript (Fig. S19). The DNA plasmids carrying the target enzymes (NOX, FDH, and ADH) were sourced from previous research projects^{44,50,51}. All plasmids were transformed by heat shock into *E. coli* BL21(DE3)Gold competent cells. For the expression of all the CAHS proteins (CAHS6), *E. coli* cells were grown aerobically in LB medium supplemented with 50 $\mu\text{g mL}^{-1}$ kanamycin. Then, 2 mL of an overnight culture was used to inoculate 100 mL of LB medium containing 50 $\mu\text{g mL}^{-1}$ kanamycin. The cell cultures were incubated at 37°C and 150 rpm until the OD₆₀₀ reached 0.6–0.8, followed by induction with IPTG and an incubation at 20–37°C overnight (see more details in Fig. S20a). For the CAHS6 protein, several expression conditions were tested until the production of the soluble protein was achieved (Fig. S20a). The cells were harvested by centrifugation at 4,000 *g* (15 minutes, 4°C), and the cell pellets were frozen until usage. The expression and solubility of proteins were evaluated by SDS-PAGE (Fig. S18b and S20b).

CAHS protein purification

Cells were thawed and resuspended in 50 mM phosphate buffer, pH 7.5, and purification was performed by a heat-shock at 95°C for 10 min. After the suspension was cooled down (10 min, at room temperature), insoluble components were pelleted at 13,800 *g* for 10 min. The soluble fraction was collected and filtered (0.2 μm). The final purity was confirmed by SDS-PAGE, and protein concentration was determined by Bradford assay. Briefly, 5 μL of the sample were mixed with 250 μL of Bradford reagent, and after 5 min of incubation at room temperature, the absorbance (595 nm) was determined in a plate reader. BSA standards were used for a calibration curve. CAHS proteins were further purified by IMAC for CD experiments (Table S3).

Enzyme purification

NOX, FDH, and ADH were purified by IMAC as previously described (purification buffers are described in Table S3)^{44,50,51}. Briefly, cells harboring the overexpressed target protein were thawed on ice, resuspended in the respective loading buffer, and sonicated at 30% amplitude for 5 min with pulses of 30 s ON, 30 s OFF. Cell debris was pelleted by centrifugation (10,500 *g*, 30 minutes, 4°C). The supernatant

was filtered (0.45 μm) before being directly loaded onto a pre-equilibrated Ni-NTA column. After purification, pre-equilibrated PD-10 columns or Amicon Ultra Centrifugal filter were used to remove the elution buffer. The protein concentrations were determined by Bradford assay and/or absorbance at 280 nm. The following parameters were used: NOX (ϵ : 45,060 $\text{L mol}^{-1} \text{cm}^{-1}$; Mw: 51.94 kDa), FDH (ϵ : 51,465 $\text{L mol}^{-1} \text{cm}^{-1}$; Mw: 42.95 kDa), and ADH (ϵ : 33,390 $\text{L mol}^{-1} \text{cm}^{-1}$; Mw: 39.28 kDa).

Enzymatic activity assays

A spectrophotometric assay previously reported was used to assess the activity of the soluble enzymes⁵². Briefly, 190 μL of reaction mix (substrate, cofactor, and 50 mM potassium phosphate buffer pH 7.5) were added to 10 μL of enzyme solution (0.03–0.5 mg mL^{-1}) in a 96-well plate. Each protein was evaluated singularly: FDH: 100 mM sodium formate, 1 mM NAD^+ at 25 $^{\circ}\text{C}$; NOX: 0.2 mM NADH, 0.001 mM FAD at 25 $^{\circ}\text{C}$; ADH: 100 mM isopropanol, 1 mM NAD^+ at 30 $^{\circ}\text{C}$. NADH formation or depletion was followed by measuring the decrease in absorbance at 340 nm for 2–20 min (according to enzyme concentration) using a CLARIOstar Plus plate reader (BMG Labtech). Measurements were performed at least in triplicate. One unit of activity was defined as the amount of enzyme needed to produce or consume 1 μmol of NADH per minute, based on the extinction coefficient difference ($\epsilon = 6,220 \text{ M}^{-1} \text{cm}^{-1}$)⁵².

Visual analysis of gelation as a function of protein concentration

To prepare samples at the desired concentrations, pre-warmed 50 mM phosphate buffer (pH 7.5) was added to dried aliquoted protein samples. The resulting mixture was heated to 95 $^{\circ}\text{C}$ for 10 min (or until complete dissolution of the protein was confirmed by visual inspection). Subsequently, 50 μL of the hot solution were transferred into NMR tubes and allowed to equilibrate at room temperature for 30 min. Tubes were then loaded horizontally and photographed. Gelation was assessed visually.

Turbidity assay

Protein samples were prepared at various concentrations (0.5–2.5 mg mL^{-1}) in 50 mM phosphate buffer (pH 7.5). The mixtures were heated at 95 $^{\circ}\text{C}$ for 10 min until fully dissolved, as confirmed visually. Immediately after dissolution, the hot samples were dispensed into 384-well microplates and allowed to equilibrate to room temperature over 1 h. Optical density (OD) measurements at 595 nm were performed using a CLARIOstar Plus plate reader (BMG Labtech). All measurements were conducted in triplicate.

Thioflavin T (ThT) fluorescence assay

Protein samples were prepared at various concentrations (20–60 μM) in 50 mM phosphate buffer (pH 7.5). Aliquots of 10 μL of each protein solution were mixed with 10 μL of 20 μM ThT solution in 384-well black, flat-bottom plates. The mixtures were incubated in the dark at room temperature for 15 min. ThT fluorescence was subsequently measured at 482 nm using a CLARIOstar Plus plate reader (BMG Labtech). Each measurement was performed in triplicate.

Desiccation experiments

100–160 μL of protein samples containing the corresponding enzyme and CAHS protein in 50 mM phosphate buffer pH 7.5 were split in half. One half was desiccated by using the SpeedVac Vacuum Concentrator for 16 h, unless otherwise specified. Rehydration was performed by the addition of 50–80 μL of H_2O . The other half was kept in the fridge as a control. Then, the remaining enzyme activity was determined following the assays described above.

Circular dichroism (CD)

Protein secondary structure was determined by using a Jasco J-815 spectropolarimeter at 25°C. The CD spectra were obtained by averaging 6 scans from 190–260 nm using a 1 mm quartz cuvette (Hellma). CAHS protein samples were prepared at 0.2 mg mL^{-1} in 50 mM phosphate buffer (pH 7.5), and to mimic water-loss conditions, trifluoroethanol (TFE) was added to final concentrations of 10–60% (v/v) followed by a 1-hour incubation at room temperature prior to CD spectral acquisition. To estimate the relative proportions of α -helix, β -sheet, and disordered region in our proteins of interest, we employed the BeStSel algorithm⁵³.

BS3 crosslinking assay

Freshly prepared bis[sulfosuccinimidyl] suberate (BS^3), an amine-reactive crosslinker, was incubated with CAHS protein at a final concentration of 1 mg mL^{-1} for 30 min at room temperature. Reaction mixtures contained BS^3 at concentrations ranging from 0.05 to 2 mM. Following incubation, loading buffer was added, and samples were heated at 95°C for 5 min. The samples were then allowed to cool to room temperature for 5 min before SDS-PAGE analysis.

Dynamic light scattering (DLS)

Dynamic light scattering (DLS) was used for a quantitative determination of the size of CAHS assemblies. Briefly, 60 μM of the different CAHS proteins were incubated for 10 min at room temperature in 50 mM phosphate buffer (pH 7.5) before measurement. Particle size distributions were determined using a Zetasizer Nano ZS (Malvern Instruments Ltd., UK) at 25°C. For each sample, three independent measurements were performed, each consisting of ten runs.

Enzyme stability tests

All stability assays were performed using NOX (at 0.58 μM) as the model enzyme, and residual enzymatic activity was used as the readout for stability under the indicated stress conditions. Where indicated, CAHS proteins were included at a final concentration of 60 μM . In experiments involving desiccation, samples were dried in a SpeedVac concentrator at 25°C for 4 h before stress exposure and subsequently rehydrated to their original volume with Milli-Q water. Enzymatic activity was determined using the standard activity assay, and retained activity was expressed relative to the initial activity of the free enzyme, which was defined as 100%.

Stability in presence of co-solvent: The stability of NOX in the presence of organic solvents was evaluated by incubating the enzyme in 50 mM phosphate buffer (pH 7.5) supplemented with 20% (v/v) organic solvent. The solvents tested were ethanol, methanol, isopropanol, acetone, acetonitrile, and dimethyl sulfoxide (DMSO). Incubations were carried out at 25°C for 1 h. Two experimental conditions were assessed: (i) direct incubation of NOX with the organic solvents, and (ii) incubation following prior desiccation of the protein samples in a SpeedVac concentrator. All reactions were performed in the presence or absence of CAHS proteins. After incubation, residual enzymatic activity was measured to determine solvent-induced loss of activity.

Stability against pH: The effect of pH on NOX stability was examined by incubating the enzyme in a universal buffer system consisting of 0.01 M citric acid, 0.01 M Tris, 0.01 M KH_2PO_4 , 0.01 M sodium carbonate, and 0.01 M KCl. The buffer was adjusted to pH 4.0, 6.0, 7.5 or 9.0. Samples were either directly incubated in the corresponding pH buffer or dehydrated prior to pH exposure. Incubations were carried out at 25°C for 20 h in the presence or absence of CAHS proteins. Following incubation, enzymatic activity was determined using the standard activity assay.

Thermal stability analysis: Thermal stability assays were performed by incubating NOX at 37°C for the indicated time intervals (0–3 h). Experiments were conducted in the presence or absence of CAHS proteins. Samples were either incubated directly or subjected to desiccation before exposure to elevated temperature, in the presence or absence of CAHS proteins. At each time point, aliquots were collected, and residual enzymatic activity was measured using the standard activity assay to evaluate the time-dependent thermal inactivation of NOX.

Storage stability assessment: Samples containing NOX (0.58 μM) were subjected to desiccation for 4 h in a SpeedVac, either in the absence or presence of CAHS6 (60 μM). Following desiccation, samples without CAHS6 were incubated for 1 h at room temperature. In contrast, samples containing CAHS6 were stored at RT for 7 or 77 days after desiccation. Following incubation, samples were rehydrated and the enzymatic activity was determined using the standard activity assay.

CAHS protein desiccation and enzyme protection: To assess the protective effect of CAHS proteins under desiccation-mimicking conditions, protein samples were dried in a SpeedVac concentrator at room temperature for 4 h. After desiccation, samples were rehydrated with Milli-Q water to restore the original protein concentration. Rehydrated samples were subsequently subjected to abiotic stress conditions, including exposure to organic solvents or incubation at different pH values, as described above. The residual enzymatic activity of NOX was then measured using the standard activity assay, allowing evaluation of the protective capacity of CAHS proteins following desiccation.

Correlation analysis - retained enzyme stability vs AlphaFold 3 predicted SSE

Retained enzymatic activity data (NOX – Fig. S10, ADH – Fig. S11) were aggregated at the level of biological replicates (2 for CAHS1-8, 1 for CAHS9; 3 technical replicates each). Data for each IDR and assay was fit to a simple two-parameter one-phase saturation model (analyze_dose_response.py) as follows:

normalized retained activity = $a \cdot (1 - e^{-k \cdot c})$ where a is a scaling parameter accounting for differences in maximum retained activity, c is the IDR concentration and k is the saturation rate. Fitting was performed on flattened biological replicates using unweighted nonlinear least squares (scipy.optimize.curve_fit) after min-max normalization in the [0, 1] range. Since profiles for different IDRs display both different maximum retained activity and saturation rates, two Area Under the Curve (AUC) metrics were used: i) data-derived, via direct trapezoidal integration making no assumption on curve shape; ii) fitting-derived, via closed-form formula:

$$\text{AUC}_{\text{fit}} = a \cdot c_{\text{max}} \cdot \left(1 - \frac{1 - e^{-k \cdot c_{\text{max}}}}{k \cdot c_{\text{max}}}\right)$$

where c_{max} is the maximum tested concentration for each IDR. AUC metrics were normalized by the c_{max} and rescaled via the ratio between the given IDR maximum retained activity and the global per-assay maximum to enable cross-protein comparisons, yielding rescaled normalized AUCs (hereon simply termed normalized AUCs).

For each CAHS N-terminal IDR, the mean (across 5 diffusion samples) total (α -helix + β -strand) number of secondary structure elements (SSE) within AlphaFold 3 predicted structures (see Fig. S9) was linearly regressed against the two normalized AUCs from pooled NOX and ADH assays (data- / fitting-derived in Fig. S12a-b). Pearson correlations were computed separately for each assay as well as for their pooled combination and reported on top of the corresponding panels.

Bi-enzymatic reaction for the synthesis of perillaldehyde

The biotransformations were carried out in 2 mL vials containing 2.5 mM of the substrate in DMSO (20% v/v), 1 U mL⁻¹ ADH, 2 U mL⁻¹ NOX, 0.1 mM NAD⁺ and 0.1 mM FAD in 50 mM potassium phosphate buffer, pH 7.5 at 30°C while shaking. To determine the yield of the enzymatic reaction, 100 μ L aliquots were quenched with 200 μ L H₂O (+ 0.2% HCl) and extracted with 200 μ L of AcOEt at different reaction times before HPLC analysis.

High-Performance Liquid Chromatography (HPLC) Analysis

The solvents used for all the HPLC analyses were 0.1% trifluoroacetic acid in water and acetonitrile. HPLC analysis was performed using a Waters Arc™ HPLC, equipped with a diode array UV – vis detector. The column used was an XBridge BEH C18, 130 Å, (3.5 μ m) (4.6 x 50 mm), 1 p/k (Waters). The following analytical method was used: flow, 0.6 mL min⁻¹; gradient, 100% acetonitrile solution held for 2 min, then gradually increase to 100% aqueous solution over 1 min, followed by an isocratic flow for 0.5 min and

gradually back to initial condition (100% acetonitrile solution) over 1.5 min to allow column re-equilibration (λ detection: 254 nm). Both substrate (rt = 0.91 min) and product (rt = 1.13 min) were analysed using this method (Fig. S21a). A calibration curve for the biotransformation product (perillaldehyde) was conducted ($R^2 = 0.9954$) (Fig. S21b). Quantification of the product in samples collected at different reaction times (from 1 to 7 hours) was performed based on the corresponding calibration curve.

Confocal microscopy

Confocal imaging was performed using a Leica SP8-AOBS-CARS confocal microscope equipped with a 63 $\times$ NA 1.4 oil immersion objective (Leica). All samples were prepared in 50 mM potassium phosphate buffer (pH 7.5) and incubated for 15 min at room temperature in 384-well plates prior to imaging. Images were processed and analyzed using ImageJ. All experiments were performed in triplicate, and a negative control was included (Fig. S14).

Analysis of CAHS6 protein self-assembly: Assemblies formed by CAHS6 were visualized by confocal microscopy under the conditions described above. Samples containing 60 μ M CAHS6 and 10 μ M ThT in 50 mM potassium phosphate buffer (pH 7.5) were incubated for 15 min at room temperature in 384-well plates. λ_{ex} : 458 nm, λ_{em} : 480–520 nm for ThT.

Colocalization assay: Confocal microscopy was employed to investigate the localization of CAHS6 and NOX in solution. NOX was labeled with rhodamine B isothiocyanate at a 1:1 molar ratio in 100 mM sodium bicarbonate buffer (pH 8.5) and incubated for 1 h at room temperature in the dark. Excess dye was removed by four sequential washes with 400 μ L of 50 mM potassium phosphate buffer (pH 7.5) using Amicon Eppendorf tubes, yielding rhodamine-labeled NOX. For imaging, NOX-rhodamine (0.1 mg mL⁻¹) was combined with CAHS6 (60 μ M) and ThT (10 μ M) in 30 μ L of 50 mM potassium phosphate buffer (pH 7.5) and incubated for 15 min at room temperature in a 384-well plate. λ_{ex} : 458 nm, λ_{em} : 480–520 nm for ThT; λ_{ex} : 561 nm, λ_{em} : 565–606 nm for Rhodamine.

NADH Fluorescence Assay

NADH fluorescence measurements were performed by incubating 20 mM NADH with 60 μ M CAHS6 in the presence of 2–8 M urea or in the absence of urea. Control conditions consisted of 20 mM NADH incubated with 2 M urea or without it. All samples were incubated for 10 min at room temperature prior to fluorescence analysis. Fluorescence spectra were recorded using a Labbot (Probation Labs, Lund, Sweden) in quartz cuvettes at 25°C. ThT λ_{ex} : 365 nm, λ_{em} : 460 nm. All measurements were performed in triplicate a negative control was also included.

Declarations

Competing interests

The authors declare no competing interests.

Author contributions

A.I.B.M. conceived the project idea and secured the funding. M.P.S. performed the experimental work with contributions from M.G.G. and A.I.B.M. M.P.S. and G.F. carried out bioinformatic and correlation analysis. All authors interpreted the data and discussed the results. M.P.S. and A.I.B.M. wrote the manuscript with contributions from G.F., M.G.G., and P.A. All authors discussed and agreed on the final version of the manuscript.

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Data availability

The data that support the findings of this study are available from the corresponding author upon request. Source data are provided as a Source Data file. Scripts for bioinformatic analysis, including AlphaFold 3 Server prediction results (zip archives), are available on GitHub (https://github.com/GiacomoFabrini/analysis_tardigrade_cahs_idrs - the repo will be made public prior to publication).

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Figures

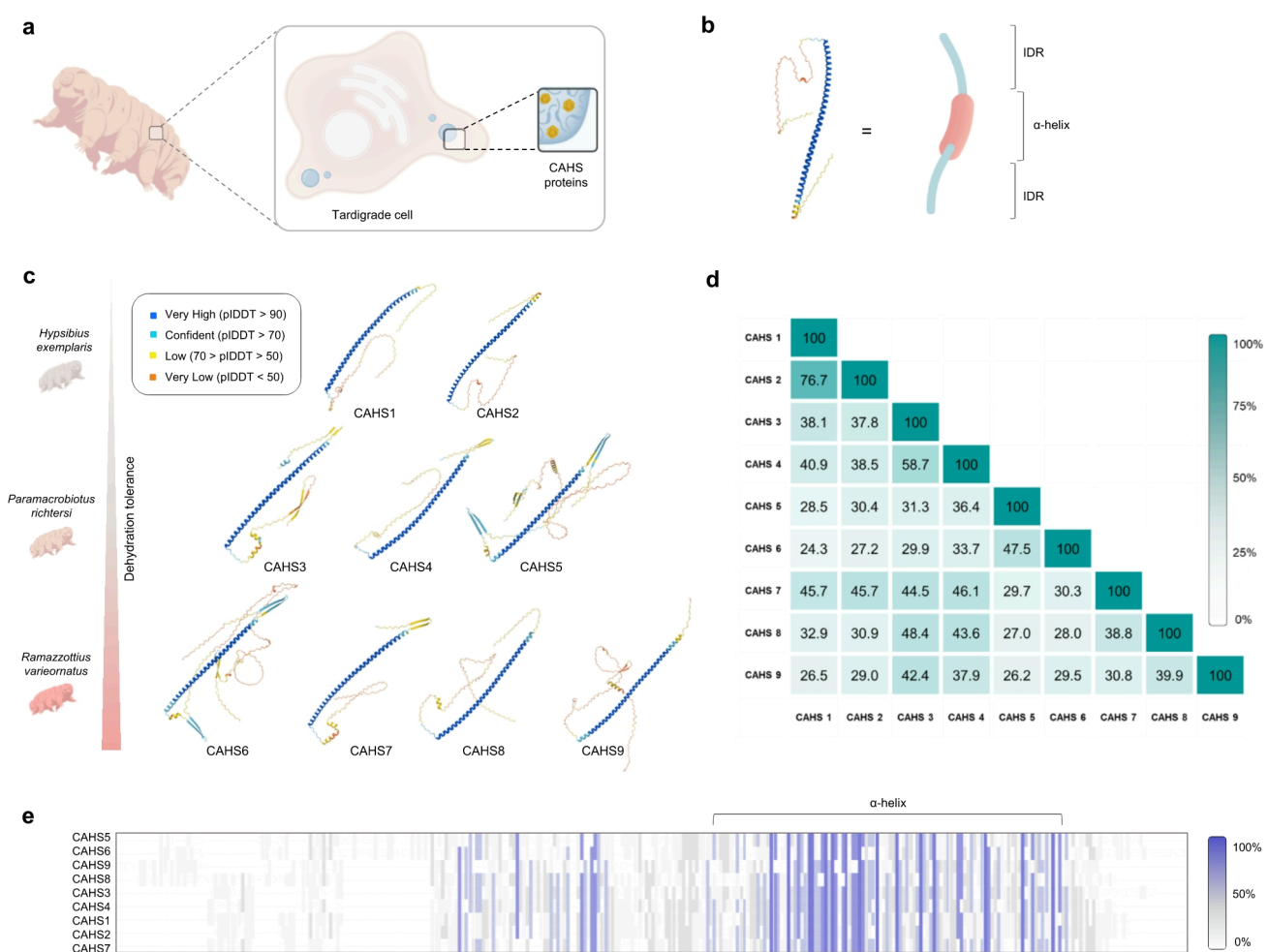


Figure 1

Sequence and structure analysis of CAHS proteins. **a)** Schematic representation of a tardigrade and subcellular localization of Cytoplasmic Abundant Heat Soluble (CAHS) proteins. **b)** AlphaFold 3 structure prediction of CAHS2 protein and schematic representation. **c)** Structure prediction of CAHS proteins using AlphaFold 3 (the corresponding UniProt IDs / Gene names are provided in Table S1). The tardigrade species from which the proteins originate are depicted on the left. **d)** Sequence identity matrix by clustalomega.org. **e)** Residue conservation analysis across CAHS protein sequences.

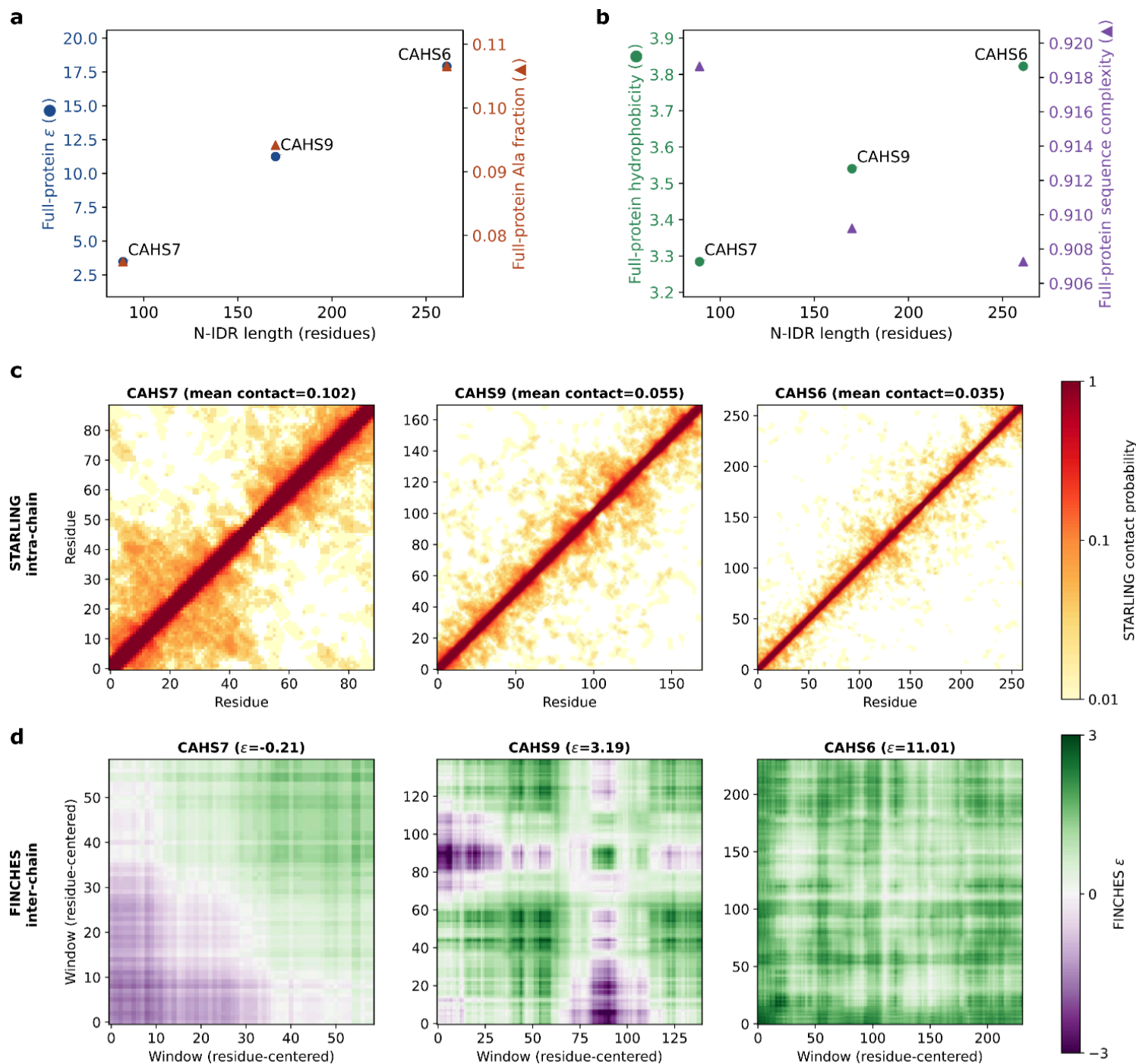


Figure 2

N-terminal IDRs of CAHS 7/9/6 span a wide range of sequence properties and intra/inter-chain behavior. a-b) Descriptors computed from full-length protein sequences vs N-terminal IDR lengths. In (a), the left y-axis shows the mean homotypic interaction strength (ϵ) computed using FINCHES³⁰ (circles), whereas the right y-axis displays alanine fraction (triangles). In (b), sequence hydrophobicity (circles) and complexity (triangles) are plotted on the left/right y-axis, respectively. Previous studies have reported higher desiccation protection for synthetic IDRs displaying higher mean homotypic interaction strength (stronger repulsion) and alanine fraction, as well as lower complexity and hydrophobicity³¹. Data for these three CAHS proteins seem to perfectly follow the first three trends, but sequence

hydrophobicity seems to go in the opposite direction. However, this might simply be due to the limited sample size. Descriptors computed from the N-terminal IDR sequences and on the entire set of investigated CAHS proteins are provided in Figure S5. **c)** Intra-chain contact maps obtained using STARLING³² (mean of 100 conformers, 150 mM ionic strength), displayed in LogNorm scale. Title reports protein name and sequence-wide off-diagonal mean contact frequency/probability (default contact threshold: 11 Å). Darker colors indicate stronger contacts. CAHS7 displays stronger/more frequent contacts, followed by CAHS9 and finally by CAHS6, being the most self-repulsive. This is consistent with trends in (a). **d)** Inter-chain interaction matrices obtained using FINCHES³³ with a default window length of 31 residues (hence the axes not perfectly mirroring the residue range in (c)). Title reports protein name and mean homotypic interaction strength (), as reported in (a). Green/purple indicates repulsion (+) / attraction (-), respectively. Once again, CAHS7 displays the most extensive attraction pattern, followed by localized attractive regions in CAHS9 and extensive repulsion in CAHS6.

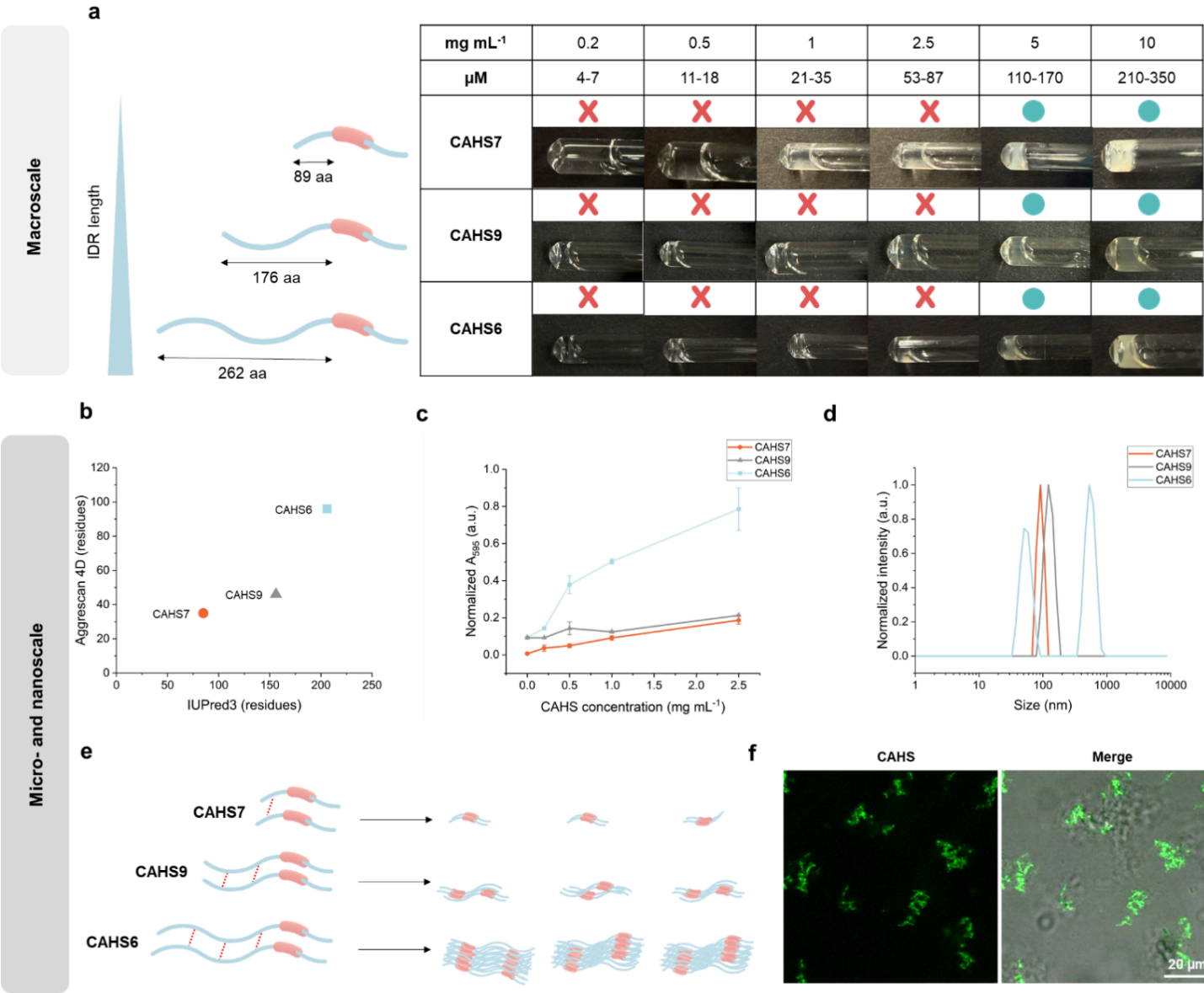


Figure 3

Biophysical characterization of CAHS protein assemblies. **a)** Macroscale formation of CAHS hydrogels. Samples forming visible gel formation are indicated with a turquoise circle and those that do not gelate are marked with a red cross. **b)** Correlation between the number of disordered residues predicted by IUPred3 and the aggregation propensity predicted by Aggrescan4D for the different CAHS proteins. Full analyses are shown in Fig. S2 and Fig. S6. **c)** Turbidity assay by measuring $A_{595\text{nm}}$. Data represent mean values from at least three independent replicates. **d)** Dynamic light scattering (DLS) intensity profiles of CAHS proteins at 2.7 mg mL^{-1} ($60 \mu\text{M}$). **e)** Schematic representation of the formation of supramolecular nanoassemblies whose size correlates with the IDR length. **f)** Confocal microscopy image of CAHS6 micro-assemblies at 2.7 mg mL^{-1} . From left to right: ThT and merged image (ThT + bright field). ThT fluorescence was detected using λ_{ex} : 458 nm, λ_{em} : 480-520 nm.

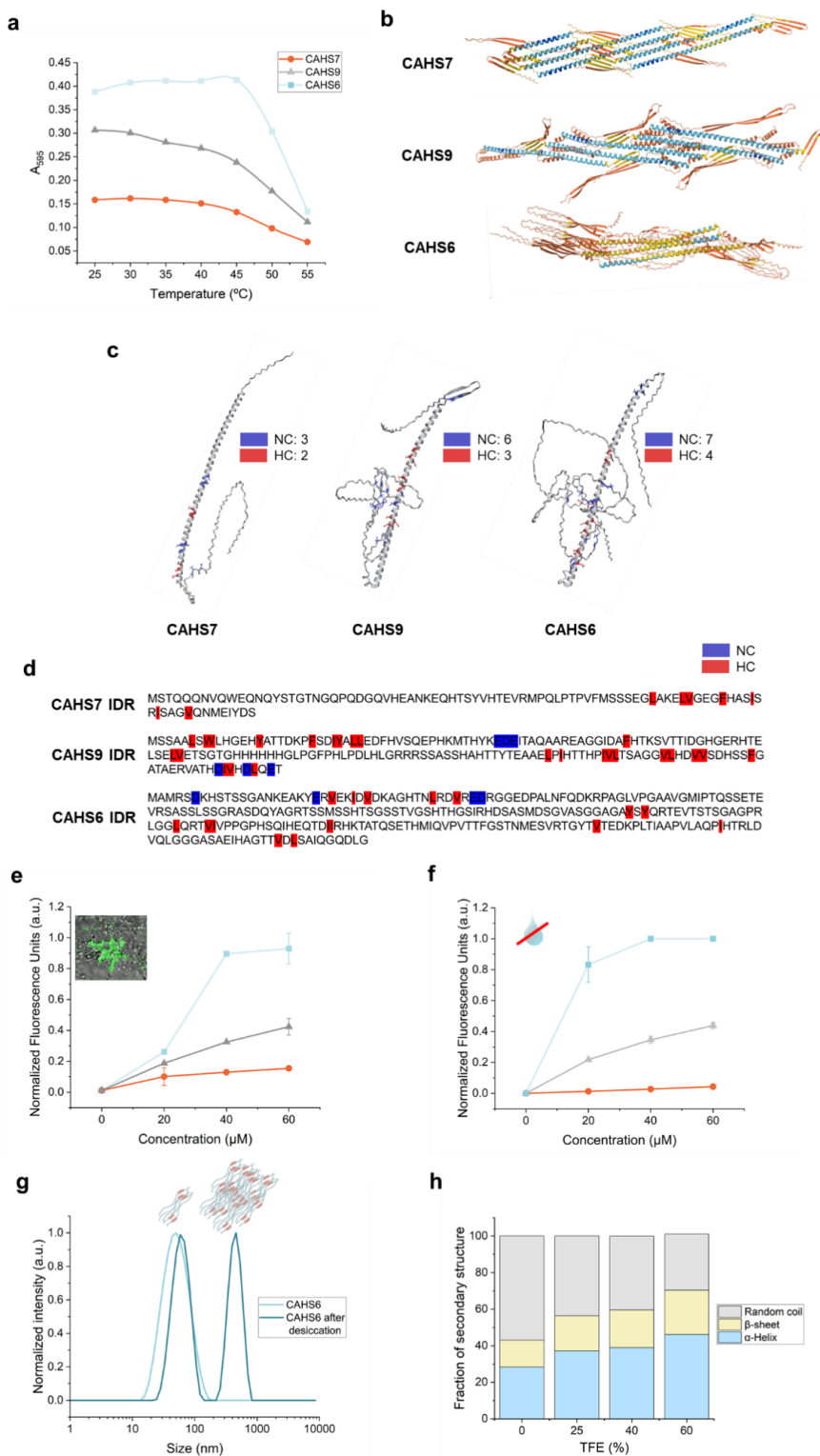


Figure 4

Protein-protein interactions during CAHS assembly. **a)** Thermal stability of CAHS aggregates measured at 595 nm. **b)** AlphaFold 3 Multimer predictions of CAHS protein assemblies. **c)** and **d)** Cluster prediction in CAHS proteins and IDRs by CapiPy. Negative clusters, NC (red) and hydrophobic, HC (blue) residues within a 10 Å distance are highlighted. **e)** ThT fluorescence assay of CAHS proteins. **f)** Same as in e) but following a desiccation-rehydration cycle. Fluorescence intensities were normalized. **g)** Intensity DLS

profiles of CAHS6 (20 μM) before (light blue) and after (dark blue) a desiccation-rehydration cycle. **h)** CD spectroscopy analysis of CAHS6. CAHS6 was incubated with increasing concentrations of trifluoroethanol (TFE; 0 to 60%). The plot shows the fractional content of the different secondary structure elements as a function of TFE concentration. All data represent the mean values from a minimum of three independent replicates.

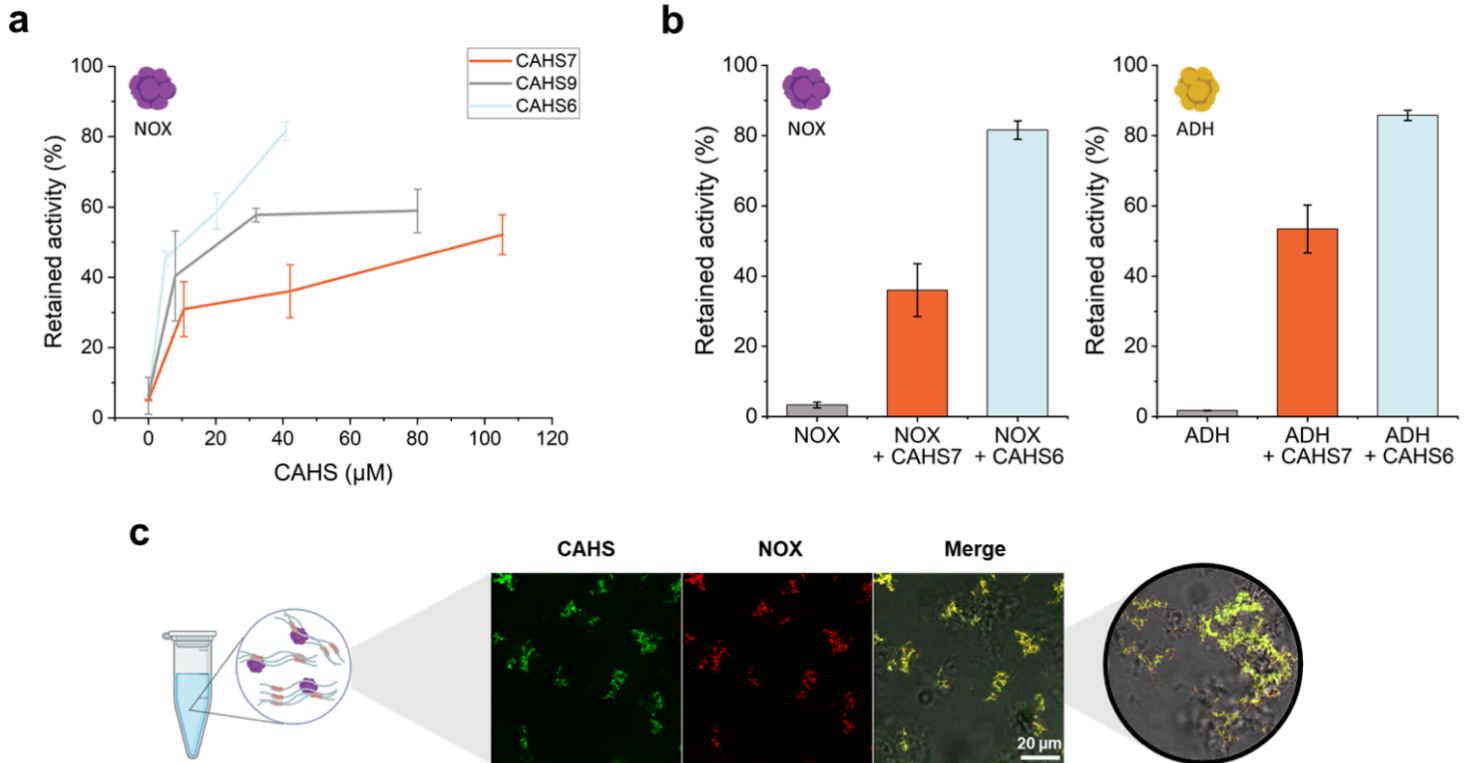


Figure 5

Effect of CAHS proteins on NOX desiccation tolerance. a) Concentration-dependent effects of CAHS7, CAHS9 and CAHS6 on NOX (0.03 mg mL^{-1}) stability. **b)** Retained enzymatic activity of NOX and ADH following incubation with CAHS7 or CAHS6 at 40 μM . Retained activity is defined as the percentage of enzymatic activity remaining after exposure to the desiccation-rehydration. Data represent mean values from at least three independent replicates. **c)** Colocalization images by confocal fluorescence microscopy. From left to right: ThT-bound CAHS6 assemblies, rhodamine-labelled NOX, and merged channels with bright field. Images of negative controls are shown in Fig. S14.

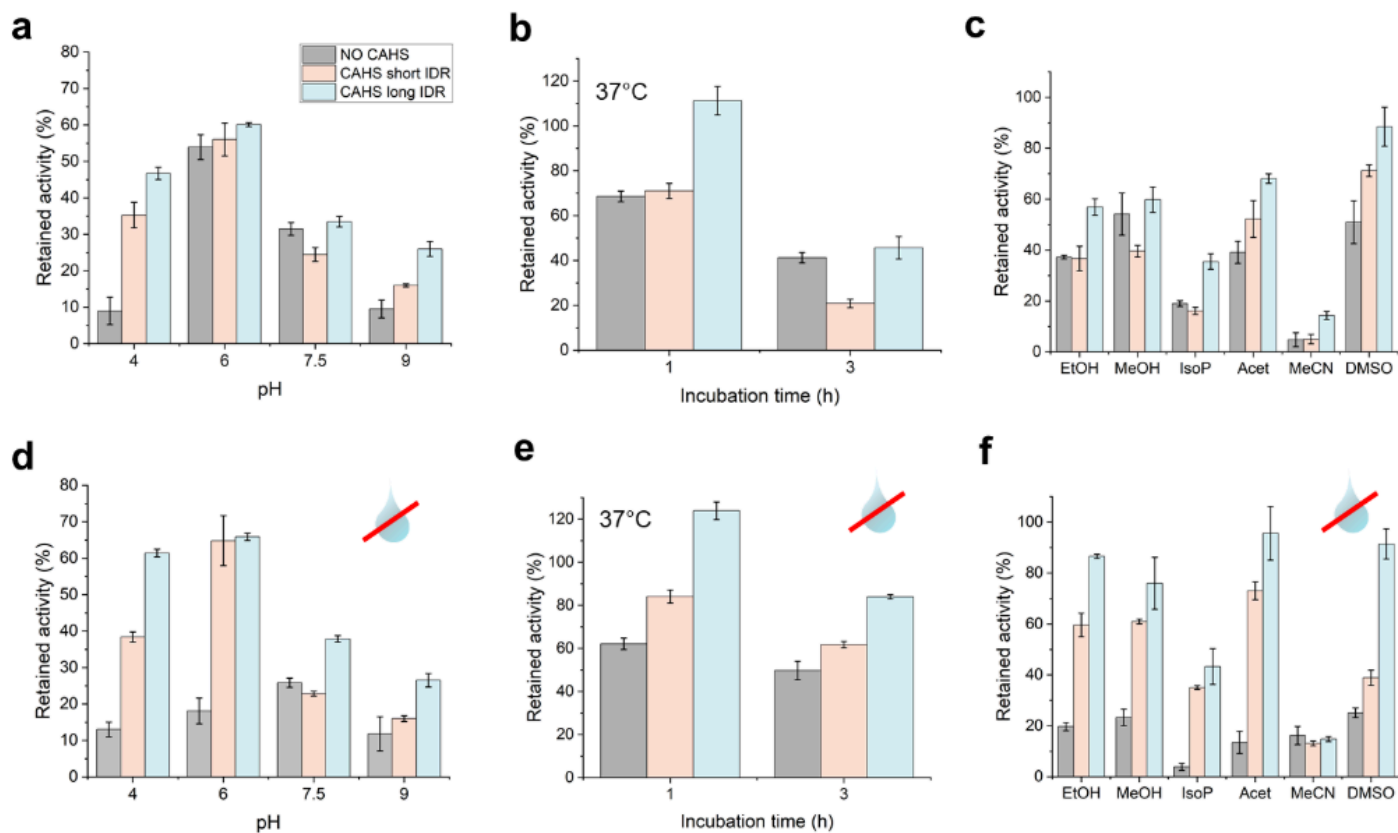


Figure 6

Stabilization of NOX by CAHS proteins under diverse abiotic stresses. **a)** pH stability. **b)** Thermal stability. **c)** Solvent stability. **d), e), and f)** show the same assays in a), b), and c) but following a desiccation-rehydration cycle. In all experiments, the retained activity was calculated relative to the initial activity measured at time zero. The data represent mean values from at least three independent replicates.

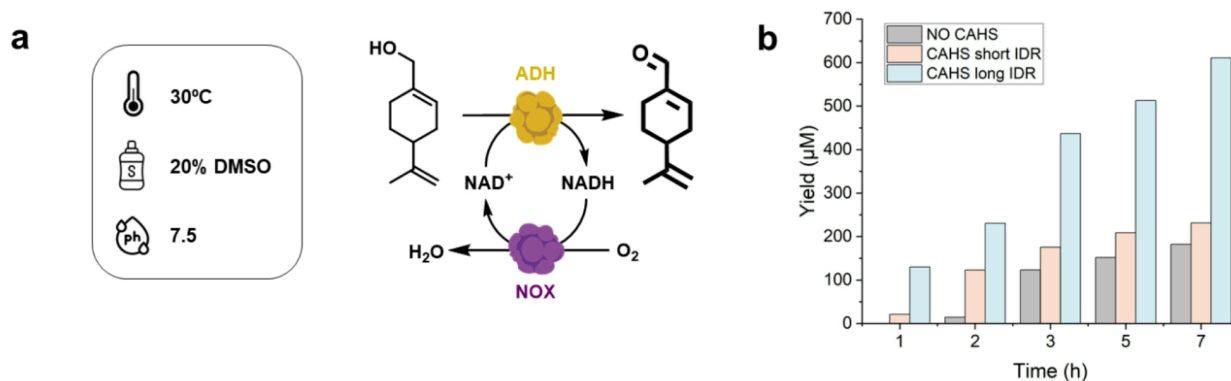


Figure 7

Enhanced bi-enzymatic synthesis of peryllaldehyde by CAHS assemblies. **a)** Schematic representation of the bi-enzymatic cascade under stress conditions for NOX and ADH (high temperature, addition of organic co-solvent, pH higher than optimal, and long operational times). **b)** Production of perillaldehyde monitored by RP-HPLC. Reaction mixture: 2.5 mM perillyl alcohol, 20% DMSO (v/v), 0.1 mM NAD⁺, 0.1 mM FAD in 50 mM potassium phosphate buffer at pH 7.5.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [RawDataExcel.xlsx](#)
- [ESICAHStabilityenzymes.docx](#)