Metabolic regulation by a catalytically inactive enzyme homolog

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Research objective

Coenzyme A transferase (CoAT) catalyzes the ATP-independent reversible transfer of CoA moieties between acyl-CoA and free acids. In intestinal bacteria, CoAT contributes to the production of short-chain fatty acids, whereas in mammals, succinyl-CoA:3-ketoacid CoAT participates in ketone metabolism, highlighting the central role of this enzyme in fatty acid metabolism. Therefore, elucidating the regulatory mechanisms of CoAT activity is important for understanding metabolic control.

In the hyperthermophilic bacterium *Thermus thermophilus*, CoAT is highly acetylated, suggesting the regulatory mechanism involves protein acetylation. Our previous studies have revealed that CoAT interacts with a catalytically inactive enzyme homolog (pseudoenzyme), alanine dehydrogenase-like protein (ADLP), which functions as a sensor of the NAD^+/NADH ratio, thereby modulating CoAT activity in an NAD^+/NADH-dependent manner. In the present study, we focused on this novel pseudoenzyme-mediated regulatory mechanism, aiming to elucidate its structural basis and physiological significance.

Methods

Structural analysis of the CoAT·ADLP complex

To reveal the structural basis of CoAT regulation via its interaction with the pseudoenzyme, we analyzed the structure of the CoAT·ADLP complex. Recombinant CoAT and ADLP were isolated from *T. thermophilus* and reconstituted as a complex. As ADLP inhibits CoAT activity upon NAD^+ binding, we attempted structural determination in the presence and absence of NAD^+ using X-ray crystallography and cryo-electron microscopy (cryo-EM). Additionally, isothermal titration calorimetry (ITC) was used to analyze the effect of NAD^+ on the interaction between CoAT and ADLP.

***In vivo* analysis of ADLP-mediated CoAT regulation**

ADLP binds to both NAD^+ and NADH with different affinities, thereby regulating CoAT activity in an NAD^+/NADH-dependent manner. To assess the physiological relevance

of this mechanism, we constructed Δ*CoAT* and Δ*ADLP* strains with the respective genes knocked out and analyzed their growth. To determine whether ADLP interacts with proteins other than CoAT and modulates their functions in a redox state-dependent manner, we generated an *ADLP*-overexpressing strain with an affinity tag for analysis in pull-down assays.

Results

Structural basis of CoAT regulation by ADLP·NAD$^+$

Because initial attempts to determine the structure of the CoAT·ADLP complex using X-ray crystallography failed, we determined the crystal structure of ADLP alone. A typical alanine dehydrogenase is a hexamer, the monomers of which comprise substrate- and nucleotide-binding domains that adopt a closed conformation upon NAD(H) binding. Similarly, ADLP forms a hexamer and contains a conserved NAD$^+$-binding site. A comparison of NAD$^+$-bound and ligand-free structures revealed that NAD$^+$ binding induces an open conformation, suggesting its role in the interaction of ADLP with CoAT and regulation thereof (Figure 1).

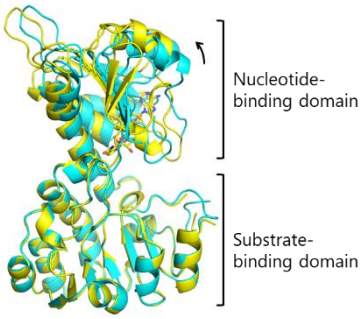

Figure 1. Conformational changes in the ADLP structure (yellow, NAD$^+$ bound; cyan, ligand free).

Cryo-EM analysis of the complex structures resolved the NAD$^+$-bound and ligand-free forms at 2.26 and 2.69 Å resolution, respectively (Figure 2). In both structures, the ADLP hexamer occupies the central position and is surrounded by CoAT dimers. The major difference is the number of CoAT dimers bound: NAD$^+$-bound ADLP clearly accommodates two CoAT dimers, with a partial model for a third, whereas the ligand-free form can only be modeled with one CoAT dimer. ITC analysis confirmed that the binding affinity of ADLP for CoAT was substantially enhanced in the presence of NAD$^+$ (K_d = 34 nM vs. 807 nM).

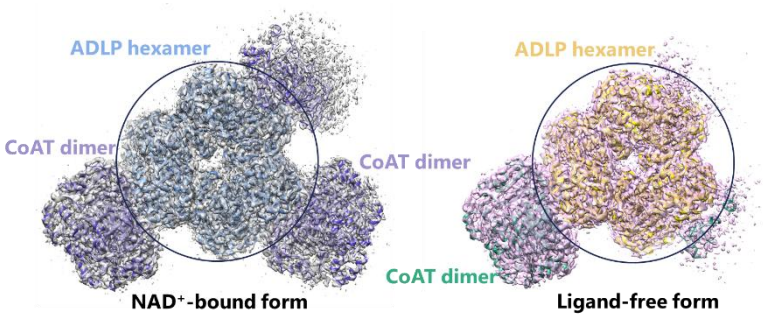

Figure 2. Cryo-EM structures of the CoAT·ADLP complex.

The ADLP hexamer consists of three dimers, each interacting with a CoAT dimer. Notably, a loop in CoAT inserts into a cleft between the substrate- and nucleotide-binding domains of ADLP. The cryo-EM structures also showed the conformational change in ADLP induced by NAD$^+$, supporting its stabilization of the ADLP–CoAT interaction. The overall structures of CoAT in the two complex forms are essentially the same, suggesting that the conformational flexibility required for catalysis is restricted by the stable interaction with

ADLP, thereby inhibiting enzymatic activity.

In vivo functional analysis of CoAT and ADLP

To assess the physiological relevance of these molecules, the growth of the Δ*CoAT* and Δ*ADLP* strains was analyzed. Δ*CoAT* growth on minimal medium with butyrate as the sole carbon source was defective, whereas Δ*ADLP* showed no significant growth difference compared with the wild-type strain under the tested conditions. Because ADLP functions as an NAD^+/NADH sensor, we may be able to observe phenotypes under conditions that alter this ratio. In the pull-down assays, no binding partners other than CoAT were detected, indicating that ADLP acts primarily to regulate CoAT.

Conclusion

This study uncovered a novel structural mechanism by which the pseudoenzyme ADLP regulates CoAT activity in response to the NAD^+/NADH ratio. NAD^+ binding induces structural changes in ADLP that stabilize its interaction with CoAT, thereby reducing the conformational flexibility of CoAT and inhibiting its enzymatic activity. We have previously found that acetylation of the CoAT protein alleviates its NAD^+-dependent inhibition by ADLP. These results suggest that ADLP modulates the acyl-CoA flux in the β-oxidation pathway by regulating CoAT activity in response to the NAD^+/NADH ratio and acetyl-CoA levels, contributing to the control of cellular fatty acid and energy metabolism. This highlights the importance of CoAT as a key metabolic node and suggests that similar regulatory mechanisms may exist in other organisms.

Furthermore, this study provides an example of a pseudoenzyme that retains its substrate- and cofactor-binding capacities despite the loss of its catalytic activity and functions as a regulatory protein for other enzymes. Combined with our previous report on the regulation of glutamate dehydrogenase by leucine and AMP mediated by two pseudoenzymes in *T. thermophilus*,[1)] the findings of this study suggest that pseudoenzyme-mediated regulation of metabolic enzymes is a widespread mechanism. Future exploration of pseudoenzymes as regulatory factors is expected to reveal previously unrecognized mechanisms of metabolic control.

References

Tomita, T., Matsushita, H., Yoshida, A., Kosono, S., Yoshida, M., Kuzuyama, T., and Nishiyama, M. (2019) Glutamate dehydrogenase from *Thermus thermophilus* is activated by AMP and leucine as a complex with catalytically inactive adenine phosphoribosyltransferase homolog. *J. Bacteriol.* **201:** e00710-18.