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Studying an orphan disease by a biophysical approach: The case of cblC

S. VILASI(1)(*) on behalf of L. LONGO(1)(3), L. RANDAZZO(1), M. BOLLATI(1), R. CARROTTA(1), M. A. COSTA(1), M. DE ROSA(1), M. R. MANGIONE(1), V. MARTORANA(1), M. MILANI(1), M. G. ORTORE(2), M. TUTONE(3) and R. PASSANTINO(1)

(1) *Istituto di Biofisica, Consiglio Nazionale delle Ricerche - Palermo, Italy*
(2) *Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche Ancona, Italy*
(3) *Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Università di Palermo - Palermo, Italy*

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Summary. — A rare disease, as defined by the European Union, is one that affects fewer than 5 persons per 10000, predominantly children. These conditions often lack effective treatments and are considered orphan diseases. Despite their rarity, there are numerous rare diseases, sharing origin and common underlying molecular mechanisms. Focusing on the metabolic rare disease cblC, we demonstrate how an approach based on biophysical methods, can pave the way for exploring novel treatment avenues for such conditions.

1. – Introduction

Methylmalonic aciduria and homocystinuria, cblC type, is a rare disease caused by mutations in the gene codifying for MMACHC, a protein involved in vitamin B12 (cobalamin, Cbl) metabolism [1]. MMACHC plays a crucial role in removing the upper axial ligand from various Cbl forms (AdoCbl, MeCbl, CNCbl, OHCbl), assisted by cofactors like reduced glutathione (GSH) for dealkylation [2, 3]. This process impairment leads to pulmonary and neuro-cognitive issues, along with degenerative maculopathy [4]. For the prevalent cblC allelic variant, c.271dupA, a frameshift mutation leading to nonsense-mediated mRNA decay (NMD) [1], gene therapy or enzyme replacement appears as the only option. However, if the MMACHC protein is produced by a specific mutated gene, less intensive strategies like pharmacological approaches could be considered. In order to do this, it is crucial to assess the impact of each specific mutation on the resulting protein structure/activity. Despite the availability of the wild-type protein (WT) crystal

(*) Corresponding author. E-mail: silvia.vilasi@cnr.it

structure [3], various molecular mechanisms related to its structure and stability still need full understanding. We used a biophysical approach to fill these gaps by answering to the following questions: are the resulting mutant proteins able to correctly fold? How much are they stable? Are they able to bind cofactors such as Cbls? Answering these questions is the initial step in designing therapeutic strategies for cblC and, in general, for similar conditions.

2. – Main steps of the biophysical approach

Our methodological approach was based on obtaining target proteins, like recombinant human wt MMACHC (MMACHC-WT) and mutants, with high yield and purity. MMACHC-WT was expressed in *E. Coli* and purified from cell lysate soluble fraction [5]. Point mutants like MMACHC-R161Q were similarly purified [6]. For mutants insolubly expressed, like MMACHC-R132X from the nonsense mutation $c.394C > T$, proteins were purified from inclusion bodies [5]. Once obtained the proteins, we investigated their stability, structure, and ability to bind and transform Cbls.

2˙1. *Study of the proteins structure and stability.* – We used Circular Dichroism (CD) to assess the secondary structure of the proteins. The MMACHC-WT FAR-UV CD spectrum, characterized by typical minima at 208 and 222 nm, agrees with its crystallographic structure [3], indicating a predominant α–helical conformation (fig. 1).

From the CD mean residue ellipticity (MRE) at 222 nm, it is possible to estimate the α-helical content by the following relationship [7]:

$$\alpha - helical(\%) = \frac{[\theta]_{222} + 2340}{3030}.$$

Similarly, one can assess whether pathologically mutated proteins differ in secondary structure compared to the wild-type (wt) protein. While the α–helical content of MMACHC-WT only slightly differs (4%) from that in the presence of Cbl (fig. 1(b)), the truncated R132X protein shows reduced α–helix compared to the first 132 residues of the wt protein [5]. MMACHC-WT's α–helical content decreases with increasing temperature, undergoing cooperative unfolding at a midpoint of 40 °C. This shift towards higher values in the presence of Cbl indicates the ligand's stabilizing role (fig. 2(a)).

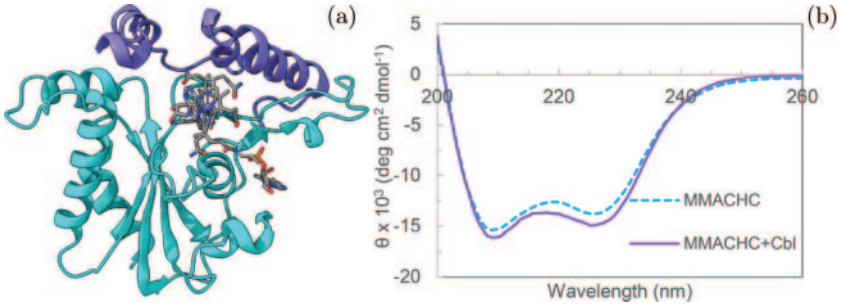

Fig. 1. – (a) MMACHC-AdoCbl (PDB 3S0M [3]) visualized by Chimera [8]; (b) CD spectra of MMACHC (17 μM) with (continuous) and without (dashed) equimolar AdoCbl.

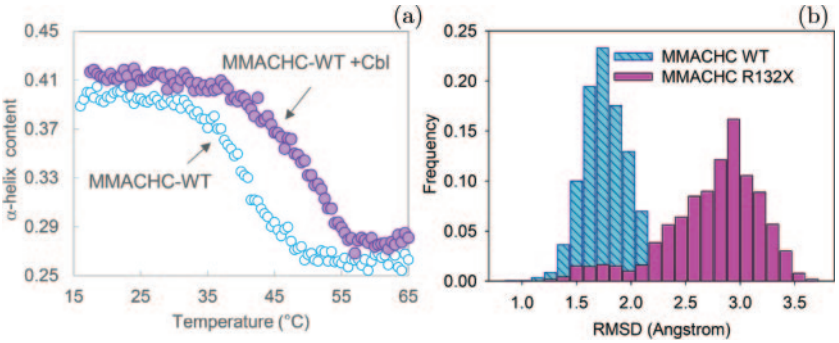

Fig. 2. – (a) α-helical content *vs.* temperature for 17 μM MMACHC without (empty) and with equimolar AdoCbl (full) from CD spectra (scan rate 0.5°C/min). (b) RMSD-values frequency distribution from 100 ns MD simulations of MMACHC-WT and MMACHC-R132X [5].

As shown by spectroscopic and computational experiments, MMACHC stability is impaired by some pathological mutations found in cblC affected patients [5, 6]. For instance, Molecular Dynamics simulations demonstrated substantial differences in root-mean-square deviations (RMSD) between the truncated protein R132X and the wt over a 100 ns simulation period (fig. 2(b)) [5].

2˙2. *Assessing the proteins ability to bind and transform Cbl.* – The increase in melting temperature for MMACHC induced by Cbl (fig. 2) serves as an indirect tool to verify vitamin-MMACHC interaction. Structural changes in MMACHC induced by Cbl, as observed by CD (fig. 1) and Small Angle X-Ray Scattering (SAXS) [5], further indicate ligand binding. However, when dealing with Cbls, a convenient method for verifying their interaction to proteins involves monitoring the consequent transitions in their structure. Specifically, when bound to some enzymes such as Methionine Synthase (MS) or MMACHC [5, 6, 9], Cbls undergo a base-on to base-off transition, which results in Dimethylbenzimidazole (DMB) tail dissociating from the cobalt atom and a change in spectroscopic features. For example, we found that MeCbl absorption maximum wavelength shifted from 530 nm to 460 nm in the presence of MMACHC protein (fig. 3(a)).

In the absence of direct methods for quantitatively evaluating Cbls binding to

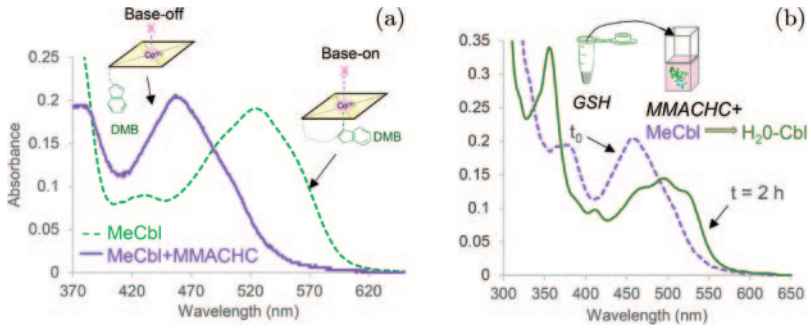

Fig. 3. – (a) Absorbance spectra of 18 μM MeCbl without (dashed) and with 22 μM MMACHC-WT (continuous) as due to Cbl base-on to base-off transition. (b) MeCbl+MMACHC absorbance spectra immediately (dashed) and after 2 h (continuous) from the 1 mM GSH addition.

MMACHC, intrinsic fluorescence quenching experiments can be employed. In fact, we compared AdoCbl dissociation constants for MMACHC-WT and MMACHC-R132X by measuring the progressive reduction in tryptophan (TRP) fluorescence with increasing Cbl amounts [5]. The mutant MMACHC-R132X exhibited a fourfold lower affinity for AdoCbl compared to the wild-type protein.

However, caution is necessary when ligands like Cbls absorb light at TRP excitation or emission wavelengths, causing an inner filter effect. This effect can be corrected using the Cbl absorbance spectrum [5] or, to avoid additional errors from differing absorption and fluorescence experiment setups, by titrating Cbl into free TRP analogs [10].

Also the MMACHC's key function, *i.e.*, the Cbl upper ligand removal, can be spectroscopically assessed. Specifically, when GSH was added to MMACHC with MeCbl, the methyl group was removed [3] from MeCbl that converted into OH_2Cbl and a characteristic absorbance maximum appeared at 350 nm (fig. 3(b)).

3. – Conclusions

We demonstrated that a biophysical approach enables to characterize the structural-functional properties of an enzyme involved in a severe rare disease. This aids in understanding which are the chemical/physical "defects" induced by pathological mutations that need correction to restore enzyme functionality. Biophysical findings, although they require *in vivo* validation, can guide focused clinical investigations. A direct application is exploring new therapeutic strategies like the use of protein stability/folding pharmacological correctors. In a broader context, this approach may benefit other conditions with shared origin and molecular mechanisms.

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REFERENCES

[1] Lerner-Ellis J. P., Anastasio N., Liu J., Coelho D., Suormala T., Stucki M., Loewy A. D., Gurd S., Grundberg E., Morel C. F., Watkins D., Baumgartner M. R., Pastinen T., Rosenblatt D. S. and Fowler B., *Hum. Mutat.*, **30** (2009) 1072.
[2] Kim J., Gherasim C. and Banerjee R., *Proc. Natl. Acad. Sci. U.S.A.*, **105** (2008) 14551.
[3] Froese D. S., Krojer T., Wu X., Shrestha R., Kiyani W., von Delft F., Gravel R. A., Oppermann U. and Yue W. W., *Biochemistry*, **51** (2012) 5083.
[4] Ricci D., Martinelli D., Ferrantini G., Lucibello S., Gambardella M., Olivieri G., Chieffo D., Battaglia D., Diodato D., Iarossi G., Donati A. M., Dionisi-Vici C., Battini R. and Mercuri E. M., *J. Inherit. Metab. Dis.*, **43** (2020) 367.
[5] Passantino R., Mangione M. R., Ortore M. G., Costa M. A., Provenzano A., Amenitsch H., Sabbatella R., Alfano C., Martorana V. and Vilasi S., *Biochim. Biophys. Acta Proteins Proteom.*, **1870** (2022) 140793.
[6] Froese D. S., Healy S., McDonald M., Kochan G., Oppermann U., Niesen F. H. and Gravel R. A., *Mol. Genet. Metab.*, **100** (2010) 29.
[7] Chen Y. H., Yang J. T. and Martinez H. M., *Biochemistry*, **11** (1972) 4120.
[8] Meng E. C., Goddard T. D., Pettersen E. F., Couch G. S., Pearson Z. J., Morris J. H. and Ferrin T. E., *Protein Sci.*, **32** (2023) e4792.
[9] Sukumar N., *Biochimie*, **95** (2013) 976.
[10] Yammine A., Gao J. and Kwan A. H., *Bio. Protoc.*, **9** (2019) e3253.