that it is highly positively charged, whereas CATR and the substrates ADP and ATP are negatively charged molecules at neutral pH. The chemical shift perturbations could represent non-specific interactions of CATR or ADP with AAC3 promoted by the high concentrations and temperatures used in these NMR experiments. We are also concerned about the validity of the dynamics studies measured by NMR relaxation dispersion, as CATR binding should lock the protein in a non-dynamic, aborted state, which is why we could solve its structure by crystallography[3]. We also note that the observed dynamics in the presence of substrate do not provide a plausible structural mechanism for transport.

We have previously demonstrated that DPC is harsh enough to solubilize unfolded mitochondrial carrier protein from *E. coli* inclusion bodies and is able to denature functional, well-folded carrier protein prepared in mild non-ionic detergents[15]. In thermostability assays[15], AAC3 purified from yeast mitochondrial membranes displayed a typical protein melt curve when diluted into the mild detergent dodecyl maltoside, consistent with thermal denaturation of a folded protein (Fig. 1c). When CATR was added at a molar ratio of 1 or greater, a marked shift in the stability of AAC3 to higher temperatures was observed, consistent with earlier observations[15,16]. When the same AAC3 preparation was diluted into 3 mM DPC, a high signal was observed with no transition, showing that AAC3 in DPC is in a non-native state (Fig. 1d). In this case, CATR addition had no effect, demonstrating that there was no functional binding site. Consistent with these findings, dilution of AAC3 into DPC before reconstitution resulted in a complete loss of CATR-sensitive ADP uptake in liposomes, in contrast to control tests where the protein was diluted into dodecyl maltoside before reconstitution (Supplementary Fig. 2). These observations clearly demonstrate that AAC3 is soluble but in a non-native state in DPC. In conclusion, we believe that the data presented by Brüschweiler et al.[1] have no biological relevance. ❐

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Published online: 3 September 2018
https://doi.org/10.1038/s41594-018-0125-6

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Acknowledgements

We would like to acknowledge support by the intramural programme MC_UU_00015/1 of the Medical Research Council, UK.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41594-018-0125-6.

Reply to 'Concerns with yeast mitochondrial ADP/ATP carrier's integrity in DPC' and 'Dynamics and interactions of AAC3 in DPC are not functionally relevant'

Yang et al. reply: In their correspondence, Kurauskas et al.[1] and King et al.[2] claim that the protein we used to measure the micro- to millisecond dynamics of yeast ADP/ATP carrier (yAAC3) in our *NSMB* study[3] was not in a functional, native state and thus the data have no biological relevance.

We strongly believe that yAAC3 in dodecylphosphocholine (DPC), although suboptimally folded and unable to generate a native dissociation constant (K_d) for ligand CATR, is nevertheless in a state that can provide qualitative information that is relevant for functional investigations. A suboptimally folded state is in many ways expected for detergent-solubilized membrane proteins; the detergent could have loosened the structure, effectively making the ligand-binding site more dynamic. Even minor destabilization of the binding site can have a dramatic effect on K_d. The key question is, can we learn anything meaningful from the yAAC3 sample used for NMR analyses? NMR allows direct K_d measurements, but this does not mean that a sample must have physiological K_d to be considered suitable for structural investigations.

The authors for both correspondence argue that DPC is a harsh detergent for yAAC3 and other, related mitochondrial carriers. We found that several carriers reconstituted in DPC could generate good solution NMR spectra while showing qualitative signs of functional relevance[4,5]. NMR-based displacement titration for SCaMC led to the identification of a key acidic residue in the higher selectivity for Mg-ATP over free ATP[5]. Fatty acid titration of UCPs revealed two basic residues important for fatty acid–assisted proton transport by uncoupling proteins[4,6]. Both studies used samples prepared in ways very similar to that for yAAC3.

The thermostability results presented by King et al.[2] for yAAC3 in DPC suggest that the protein is not in a native state. The discrepancy with our studies could be due to

differences in purification protocol. In our NMR studies, the yAAC3 samples contained 0.8 mM yAAC3 and ~120 mM DPC, yielding a protein:detergent ratio of 1:150; in our isothermal titration calorimetry (ITC) samples, the protein:detergent ratio was likely higher, but we did not measure those concentrations.

In their correspondence, Kurauskas et al.[1] and King et al.[2] both argue that the CATR affinity observed for yAAC3 in DPC was orders of magnitude lower than previous reports in the literature and thus the yAAC3 in our NMR sample was not natively folded. We argue that the weakened ligand binding of membrane protein in detergent is not necessarily due to protein unfolding. If yAAC3 in DPC were unfolded, it should not bind CATR with a K_d of ~20 μM and 1:1 stoichiometry, as determined by ITC[3]. We acknowledge that the previously reported K_d of 192 μM for CATR[7] was a typographical error, which was later corrected to 192 nM. Kurauskas et al.[1] raised the possibility that our observed CATR interaction was due to non-specific electrostatic interaction, as CATR is negatively charged and yAAC3 contains many basic residues. We performed ITC experiments on CATR binding to DPC-reconstituted UCP1 and SCaMC, which are homologous to yAAC3 and also contain many basic residues, and saw no binding (Supplementary Fig. 1), indicating that CATR binding to yAAC3 in DPC is specific, albeit with a K_d of ~20 μM. The functional relevance of the UCP1 and SCaMC samples has been demonstrated in previous studies[5,6].

After reanalyzing our NMR-based CATR titration data, which showed widespread non-specific chemical shift perturbation (CSP) for the GDP/GTP carrier (GGC1, which is not supposed to bind CATR), Kurauskas et al.[1] concluded that the CATR-induced CSPs we observed in yAAC3 were also due to non-specific interactions. CATR is hydrophobic and can partition into DPC micelles. Thus, at high CATR concentrations (5–10 mM), CSPs could be induced simply by alteration of the micelle environment. The CSP values we observed are indeed below 0.1 p.p.m. (normalized to ^{1}H), but the magnitude of ligand-induced CSPs depends on perturbations in the electronic environment of protein backbone amide and are thus not proportional to any physical aspects of binding per se. We clarify that we deemed the CSP data significant as they allowed us to obtain a binding saturation curve; however, the CSPs were not interpreted on their own, but rather in conjunction with the ITC data.

Kurauskas et al.[1] showed that relaxation dispersion profiles generated using the same exchange parameters (p_e, k_{ex} and $\Delta\nu$) could fit our Carr-Purcell-Meiboom-Gill (CPMG) data for the ADP- and CATR-bound yAAC3 samples equally well. We clarify that we performed collective fitting to the exchange model using all residues that showed significant relaxation dispersion, in the standard program cpmg_fitd9[8]. We acknowledge that small uncertainties in CPMG data could potentially result in large differences in the exchange parameters[9] but cannot explain why Kurauskas et al.[1] were able to obtain a similar quality of fit using entirely different exchange parameters. Nevertheless, on the basis of visual inspection of the dispersion curves, we find that the exchange parameters between the three different states cannot be the same because their corresponding dispersion curves are very different (Supplementary Fig. 1 in ref. [1]). ❐

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Published online: 3 September 2018
https://doi.org/10.1038/s41594-018-0126-5

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Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41594-018-0126-5.