> Authors submitting altogether predictive manuscripts to established journals are typically required to demonstrate applicability. It has been assumed that submissions to the Festschrift issue of MR Discussions honoring Geoffrey Bodenhausen are also subject to this requirement.

As in our response to referee #1, we feel this notion is warranted. Still, with the given deadline, the developed concepts and the preliminary data represent the project's current status. We deem it a worthwhile contribution to the Festschrift.

> The first sentence is obvious and the objective of this work is appreciated. The issues at stake include the definition of the "segment" as conceived by Mantsyzov et al.[1] (to which the authors refer), and the physical relevance of the "measure". C^{α} and C' belong to residue $i - 1$; N and H^{N} belong to residue i. $D_{\parallel}/D_{\perp}$ are likely to differ for consecutive $(i - 1) - i$ pairs. How will then the ratios, $D_{\parallel}/D_{\perp}$, between the parallel and perpendicular components of the global diffusion tensor, $\boldsymbol{D}$, of the "segment" be determined? The "measure" suggested is ill-defined – see the original review report, and this report. On the most general level – in the Introduction the authors indicate that time-correlation functions (TCFs) developed for folded proteins, which include model-free-type TCFs, are not applicable to intrinsically disordered proteins (IDPs). Yet, they suggest a TCF of this type. With regard to these matters have I suggested considering – or at least discussing – coil libraries and molecular dynamics simulations, e.g., as in Mantsyzov et al.[1]

We feel these two general issues should be treated and highlighted separately. We will refer to these arguments throughout.

<u>Issue 1) Are we justified to invoke the concept of a tumbling symmetric top in the context of IDPs?</u>

As we emphasized before, IDPs cannot be expected to generally adhere to this model. We do not suggest a TCF of this type to model IDPs. We want to probe whether there are anisotropy features/biases in the (segmental) dynamics of (partially) disordered proteins commonly associated with this model. Studies invoking this concept in the context of (partially) disordered proteins were provided ranging from "fully disordered" to partially structured segments. IDPs are not "random coils" but a diverse class of proteins with context specific structural features.

As stated in the manuscript, isolating the J(0) components of NH^{N} CCR and $C'C^{\alpha}$ CCR provides a very general measure for the presence of anisotropic dynamics at the scale of the peptide plane even without a specific dynamic model in mind. Since we can identify the isotropic case, we can also detect deviations thereof. We only consider the simplistic and qualitative definition of a tumbling "segment" previously asserted by (Mantsyzov et al. 2014) to assess the sensitivity of Q. As emphasized in the Introduction, we are agnostic about the relevance/applicability of this simplified representation. Q could likely rule out this concept in many/most cases, it depends on the specific signature. The experiments were designed to probe the presence of anisotropic "slow" motions associated with segmental tumbling. If the J(0)s of the two spin pairs are similar, peptide plane dynamics in IDPs would appear to be isotropic and well-probed by conventional ^{15}N relaxation. If the obtained J(0)s exhibit pronounced differences, the source of anisotropy should certainly be investigated in further detail (preferably in conjunction with MD simulations). As IDPs are a diverse family of proteins, the results will necessarily depend on the system under investigation.

We do not see any inherent contradiction in assessing and illustrating the sensitivity of Q using the previously invoked concept of a symmetric top. The limited validity of this model for IDPs is emphasized throughout the paper. We can certainly emphasize these considerations and further clarify the exemplary nature of this model in the manuscript.

Issue 2) Does our proposed spectral density represent CCR of a tumbling symmetric top? Is the approximate MF-like inclusion of local motions properly handled?

Based on the objections thus far, we deem our description of the tumbling symmetric top appropriate as it is. The apparent deficiencies are addressed in detail below.

In the following, we will address the open questions and comments:

As stated in our previous response and in the original manuscript, we do not suggest that any parameters such as $D_{\parallel}/D_{\perp}$ could be "determined" as the dynamic model is too simplified. We are only comparing the J(0)s, we do not claim that solving the inverse problem, i.e. extraction of the (hypothetical) diffusion tensor, was possible or intended. That being said, why would $D_{\parallel}/D_{\perp}$ be likely to differ for consecutive peptide planes if they tumble in a concerted fashion? We would not rule out this effect, but we would speculate that the relative orientations of the peptide planes are a more relevant source of uncertainty.

MD simulations are highlighted throughout the manuscript, but we can further emphasize their potential. In the authors' opinions, MD simulations are the ideal "dynamic model" for IDPs. If the CCR rates turn out to be "heterogeneous enough", we will certainly include simulations in a future study. Limited to (φ,ψ)-space, like in (Mantsyzov et al. 2014), coil libraries can be suitable priors for structural inference (Kauffmann et al. 2021). To investigate dynamics of consecutive residues, we tend to prefer MD simulations over the static and generic nature of coil priors.

(a) The expression for τ_3^{-1} is valid in the limit where $\tau_{int}^{-1} >> \tau_{eff}^{-1}$. What is the justification for the validity of this inequality for IDPs?

See Issue 1, we are not modeling the dynamics of IDPs, we are considering a MF-like symmetric top.

(b) In the limit of isotropic global motion one has $\sum_{K=0,1,2} A_K(u,v) \frac{\tau_K}{1+(\omega\tau_K)^2} \rightarrow \frac{\tau_0}{1+(\omega\tau_0)^2}$.

Where does the angular relation between *u* and *v* go? As pointed out by referee #2 in the context of (Ghose et al. 1998) as well, in the limit of isotropic global motion one has $\Sigma_{K=0,1,2} A_K(u,v) \frac{\tau_K}{1+(\omega\tau_K)^2} \rightarrow P_2(u\cdot v)\frac{\tau_0}{1+(\omega\tau_0)^2}$. As the index "0" might be confusing for some readers, we note that as $D_{\parallel}/D_{\perp} = 1$, $\tau_0 = \tau_1 = \tau_2 = \tau_{tumb} = (6D)^{-1}$.

In that limit the authors of mr-2021-35 maintain that $(1-S^2)\,P_2(u\cdot v) \rightarrow S_{uv}^2$.

This is not correct. In that limit we state that $S_{uv}^2 \rightarrow P_2(u\cdot v)S^2$ such that $P_2(u\cdot v) - S_{uv}^2 \rightarrow (1-S^2)P_2(u\cdot v)$

$$J_{u,v}(\omega) = \left[S^2 \frac{\tau_0}{1+(\omega\tau_0)^2} + S_{uv}^2 \frac{\tau_e}{1+(\omega\tau_e)^2}\right] = \left[S^2 \frac{\tau_0}{1+(\omega\tau_0)^2} + S^2\, P_2(u\cdot v) \frac{\tau_e}{1+(\omega\tau_e)^2}\right] \qquad (2)$$

This is not the isotropic limit of Eq 1 (Eq 10 mr-2021-35). As stated above, $A_K(u,v)$ sums up to $P_2(u\cdot v)$ and we are not sure why the "auto" S^2 and the "cross" S_{uv}^2 became mixed liked this. The isotropic limit of Eq 1 (Eq 10 mr-2021-35) is exactly as required:

$$J_{u,v}(\omega) = P_2(u\cdot v)\left[S^2 \frac{\tau_0}{1+(\omega\tau_0)^2} + (1-S^2) \frac{\tau_e}{1+(\omega\tau_e)^2}\right] \qquad (3)$$

If S^2_{uv} was used throughout, we would instead obtain

$$J_{u,v}(\omega) = S^2_{uv}\frac{\tau_0}{1+(\omega\tau_0)^2} + (P_2(u\cdot v) - S^2_{uv})\frac{\tau_e}{1+(\omega\tau_e)^2}$$

(Ghose et al. 1998) Eq 9, as later referred to by referee #2, with the angular relations now contained in S^2_{uv}.

This isotropic limit is both contained in Eq 11 (mr-2021-35) and illustrated in Fig. 4 (mr-2021-35). As mentioned in the manuscript, it can be seen that the limit of fully isotropic local motions and isotropic tumbling coincides at around 0.3.

Thus, using eq A14 of ref 2 instead of eq A.15 of ref 2 does not remove the requirement that the polar angle, α, be small.

With the validity of A.14 (Tjandra et al. 1996) now established, we can agree that it represents the analytic solution for a tumbling rigid symmetric top. This is an improvement over the approximation via the auto-correlated TCF. Thus, for $S^2 = 1$, Eq. 1 (Eq. 10 mr-2021-35) is exact. For $S^2 = 0$, it is also exact. As established above, for isotropic tumbling, $D_\parallel/D_\perp = 1$, it also follows MF-type expressions as above and referenced in the manuscript/response.

In essence, Eq. 1 (Eq. 10 mr-2021-35) weights/mixes the two exact solutions via S^2. This approximation becomes better the closer S^2 is to 0 or 1, the closer $D_\parallel/D_\perp$ is to 1 and/or the closer $u{\cdot}v$ is to 1. We cannot follow at what size and combination of $u{\cdot}v$, S^2 and $D_\parallel/D_\perp$ the approximation becomes entirely unreasonable especially when considering that the only "large" angle is between C'C$^\alpha$ and σ_{yy} which contributes with smaller weight in terms of both size (σ_{yy}-σ_{zz}) and the initial value of $P_2(u\cdot v)$ (as highlighted in the manuscript). $J_{C'C\alpha,xx}(0)$ is clearly the dominant component.

We cannot see how the value of $u{\cdot}v$ for C'C$^\alpha$ and σ_{yy} might break the (anyways approximate) interpolation between rigid anisotropic tumbling and fully isotropic motions presented in Fig. 4 (mr-2021-35).

The sentence in my original report to which the authors refer follows the description of the standard treatment of cross-correlated relaxation for isotropic global diffusion. It reads: "Equations 2–4 do not comply with THIS standard NMR relaxation procedure." The authors interpret "THIS" as "THE". I admit that "do not comply with" is bad phrasing; it should have been "do not represent". By the way, the upper limits in the summations of eqs 3 and 5 of mr-2021-35 should be infinity.

This is not an issue of semantics. We still do not see how Eqs. 2-4 (mr-2021-35) do not represent very general properties of TCFs encountered in solution-state NMR. How are MF-like TCFs not contained within these boundaries?

Clearly Eq. 3 is less general than Eq. 2, one could generalize further and consider an infinite sum. We never introduced Eq. 3 as the most general description, we only responded to the claim that it does not comply with MF-type TCFs.

For most practical purposes, we do not see how the difference between large N and infinity might result in substantial discrepancies both in the context of our manuscript and with respect to TCF shapes of proteins in isotropic solution. That being said, we do value the benefits of continuous

notation. Before introducing Eq. 3, we specifically reference the concept of correlation time distributions (mr-2021-35 page 4).

> been derived by (Deschamps & Bodenhausen, 2001). Can referee #2 clarify, is the validity of Eqs. (6) and (7) (mr-2021-35) being questioned or the combination with a fourth Lorentzian in Eq. (10)?

The comment made here refers to the usage of eq A.14 instead of A.15, discussed in item (c) above.

In the first response of referee #2, we got the impression that the general notation and validity of A.14 (Tjandra et al. 1996) was being questioned. We apologize if we misunderstood.

- From (2) to (9) (mr-2021-35) we simply establish the effect of anisotropic diffusion on the TCF which depends on the relative orientations of **u** and **v**. For $H^{\alpha}H^{N}$ intraresidual and

Please see above with regard to Mantsyzov et al.[1]

We were responding to the following statement, referring to Eqs 2-9 (mr-2021-35) as problematic:

> Based on eqs 2–9, shown above to be problematic, the authors obtain the spectral density:

$$J_{u,v}(\omega) = S^2 \sum_{K=0,1,2} A_K\,(u,v)\,\frac{\tau_K}{1+(\omega\tau_K)^2} + (1-S^2)\,\sum_{K=0,1,2} A_K\,(u,v)\,\frac{\tau_3}{1+(\omega\tau_3)^2} \quad (3)$$

In case this led to confusion, it should be highlighted that this is not the proposed spectral density (Eq 10 mr-2021-35). We considered this as a transcription error.

More generally, see Issues 1 and 2.

As shown above, eq 1 is not applicable to the spin system C^{α}–C'.

As discussed above, we do not agree with this statement. In addition, the highlighted TCF of (Ghose et al. 1998) includes a similar spin system, namely C'–N. Arguably, the angular relations of C' CSA – C'N DP CCR are not too different from C' CSA – C'C^{α} DP CCR. More details below.

The local-motional contribution of $(1-S^2)\,\frac{\tau_e}{1+(\omega\tau_e)^2}$ can be justified on the basis of the theory of moments.[3] The local-motional contribution $(1-S^2)\,P_2(u\cdot v)\,\frac{\tau_3}{1+(\omega\tau_3)^2}$ is used allegedly.

We discussed in multiple paragraphs how the isotropic contribution can be justified. It is part of the reason we denote it as such as it provides a better "feel" for many. Still, we find the contribution of $P_2(u\cdot v)(1-S^2)\frac{\tau_3}{1+(\omega\tau_3)^2}$ on J(0) worth highlighting in purely mathematical terms.

The reservations expressed in the original report and here concern eq 10 of mr-2021-35. All of the articles cited in the preceding paragraph feature physically well-defined TCFs.

We were referring and contextualizing with respect to the question of referee #2: "What physical framework could possibly justify the second term of eq 3?", i.e. $P_2(u\cdot v)(1-S^2)\frac{\tau_3}{1+(\omega\tau_3)^2}$

The articles were cited to explain all components, i.e. the factor $P_2(u \cdot v)$, the use of an auto-correlated order parameter S^2 and the concept of the effective isotropic tumbling time.

Explicitly or implicitly all three MF models (A, B and C) considered by Halle[4] assume statistical independence between the global and internal motions. One may not "choose" arbitrarily an exponential form for $C_{int}(t)$; one has to justify this (e.g., see ref 3). It is shown above why using eqs 6 and 7 does not render eq 1 applicable to arbitrary polar angle, α. Equation 7 of Ghose et al.[5] does not lack the factor $P_2(u \cdot v)$; note the summation over l in it, and the evolution of this equation into eq 9 of that article. Halle[4] voices supportive assessment of four specific MF formulae, none claimed to apply to IDPs.

Generally, see Issues 1 and 2.
Specifically:

As Halle's MF-B (Halle 2009) does not prespecify the form of $C_{int}(t)$, the exponential form requires commentary. We specifically referred to the previous references to justify it.

As stated above, the effect of summation over l is not the issue. We explicitly referred to the **internal** TCF of (Ghose et al. 1998) Eq. 9 (using the previous notation):

$$J_{u,v}(\omega) = S^2 \sum_{K=0}^{2} A_k(u,v) \frac{\tau_k}{1+(\omega\tau_k)^2} + (1-S^2)\frac{\tau_e}{1+(\omega\tau_e)^2} \qquad (a)$$

Note that the index "x" for "cross" in the order parameters has been omitted for clarity. (Ghose et al. 1998) justify the above expression as follows "In order to obtain expressions analogous to conventional Lipari–Szabo theory, we express the auto- and cross-correlation spectral density functions using effective order parameters." (page 488).

Defined to be analogous to the conventional "auto" order parameters, the "effective order parameters" lie between 0 and 1. This can be seen in two ways: In the fully rigid tumbling limit $J_{u,v}(\omega) = \sum_{k=0}^{2} A_k(u,v) \frac{\tau_k}{1+(\omega\tau_k)^2}$, thus $S^2 = 1$. It can also be seen from the initial value 1 of the internal TCF.

We cannot follow the "evolution of this equation into eq 9 of that article". For isotropic tumbling, $\Sigma_{K=0,1,2} A_K(u,v) \frac{\tau_K}{1+(\omega\tau_K)^2} \rightarrow P_2(u \cdot v) \frac{\tau_0}{1+(\omega\tau_0)^2}$, we would obtain:

$$J_{u,v}(\omega) = S^2 P_2(u \cdot v) \frac{\tau_0}{1+(\omega\tau_0)^2} + (1-S^2)\frac{\tau_e}{1+(\omega\tau_e)^2} \qquad (b)$$

This is not the isotropic limit of Eq. 9 of (Ghose et al.) which reads

$$J_{u,v}(\omega) = S_{uv}^2 \frac{\tau_0}{1+(\omega\tau_0)^2} + (P_2(u \cdot v) - S_{uv}^2)\frac{\tau_e}{1+(\omega\tau_e)^2} \qquad (c)$$

with the angular relations encoded by S_{uv}^2. Using $S_{uv}^2 = P_2(u \cdot v)S^2$, we obtain the familiar

$$J_{u,v}(\omega) = P_2(u \cdot v)(S^2 \frac{\tau_0}{1+(\omega\tau_0)^2} + (1-S^2)\frac{\tau_e}{1+(\omega\tau_e)^2}) \qquad (d)$$

Importantly, we do not think (Ghose et al. 1998) are implying Eq 7 (a) evolves into Eq 9 (c). Eq 7 (a) is defined purely by analogy. Arguably, the index "x" for "cross" is used in a somewhat confusing manner. If the order parameters in Eq 7 (a) were interpreted as "proper" cross-type, one would

encounter different issues. In the fully rigid tumbling limit, the angles would be accounted for twice, i.e. it would decay from $P_2(u \cdot v)^2$ while the internal TCF would still decay from 1. One would not end up with Eq. 9 (c).

It can be seen how this not too problematic for smaller angles, but as we stated in our first response, we feel that the lack of $P_2(u \cdot v)$ for the **internal** TCF is problematic for larger angles. By having the internal TCF decay from $P_2(u \cdot v)$ towards $S_{uv}^2 = P_2(u \cdot v)S^2$, Eq. 10 (mr-2021-35) is obtained.

To be clear, we feel the expressions of (Ghose et al. 1998) are still reasonable. Analogies are a good way to make the features of an (anyways approximate) dynamic model more relatable.

> One cannot attain objectives with inadequate tools.

Regarding the sensitivity assessment and the dynamic model, we feel it is adequate, see Issues 1 and 2. The isolated J(0) components of NH^N CCR and $C'C^\alpha$ CCR probe the motions of the shared peptide plane at the same frequency. Thus, they are straightforward to compare and the expectation for isotropic motions can easily be specified. To us this is an adequate tool for detecting the presence of anisotropic dynamics.

> Comment no. 5 refers to the form of the spectral density, not the data-fitting process. One cannot detect actual effects with inappropriate tools.

We apologize for the misunderstanding. We already commented on the concept of correlation time distributions, which the approach appears to build on. However, it puts an interesting twist on it by emphasizing the timescales the relaxation parameters are most sensitive to. For now, we feel our proposed (spectral density mapping like) protocol should be appropriate for detecting actual effects without specifying the form of the spectral density. Regarding the spectral density used to assess our protocol, see Issues 1 and 2. The references are appreciated and might prove useful in future applications when it comes to interpreting the observed effects in structural/dynamical terms.

Comments (2), (3) and (4) have been addressed above.

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