Reply to the authors' rebuttal to reviewer #2

Regarding general matter **(1)**, as referee #1 has pointed out as well, our study is indeed predictive and conceptual in nature. In our paper, we want to acknowledge Geoffrey Bodenhausen's contributions to the field by discussing how CCR experiments developed in his group over the years can potentially be used to investigate and characterize local (segmental) dynamics in (partially) disordered proteins.

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.

Let us clarify: We do not claim nor intend to provide a thorough and general description of IDP dynamics. While segmental motions are clearly present in IDPs (Rezaei-Ghaleh et al. 2018, Parigi et al. 2014), it is obvious that the concept of diffusion anisotropy cannot be expected to rigorously apply. Still, this somewhat elusive concept has been invoked in previous studies even for proteins such as α-Synuclein (Mantsyzov et al. 2014). This leads us to the central question of our study: If the segmental motion of IDPs exhibited features/trends associated with anisotropic tumbling, how could we best detect it? Can we define a sensitive and unambiguous experimental measure?

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]

Complementing NH^{N} CCR, we show that $C'C^{\alpha}$ CCR allows to probe the peptide plane quite literally from a different angle. The isolated zero frequency components are straightforward to compare and particularly sensitive for larger correlation times, allowing to probe the presence or lack(!) of anisotropic dynamics in IDPs on the local scale of the peptide unit. To assess the combined information content of the CCR rates, we build on the previously invoked and simplified model of a tumbling symmetric top. The "dampening" effect of local motions is approximated as simply as possible using a single exponential decay with equal weight for both NH^{N} and $C'C^{\alpha}$. We do not claim this treatment is rigorous nor do we imply that features of this MF-like anisotropic tumbling should be expected for all protein systems. The experiments were conceptualized and designed to assess the presence of features/trends associated with this simplified dynamic model.

As presented, the axial top includes a given $(i-1)-i$ pair. Its global tumbling is represented by the first term of eq 10 of mr-2021-35. That equation is depicted below as eq 1. The "dampening" effect of the local motions is given by the second term or eq 1.

$$J_{u,v}(\omega) = S^2 \sum_{K=0,1,2} A_K\,(u,v)\,\frac{\tau_K}{1+(\omega\tau_K)^2} + (1-S^2)\,P_2(u\cdot v)\,\frac{\tau_3}{1+(\omega\tau_3)^2} \qquad (1)$$

u and v are the cross-correlated interaction vectors. One has $\tau_3^{-1} = \tau_{int}^{-1} + \tau_{eff}^{-1} = \tau_{int}^{-1} + (4\,D_\perp + 2\,D_\parallel)$. I regret my oversight in the original report, where I mistook $(4\,D_\parallel + 2\,D_\perp)$ for $(4\,D_\perp + 2\,D_\parallel)$.

I have the following reservations with regard to this equation.

(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?

(b) In the limit of isotropic global motion one has $\sum_{K=0,1,2} A_K\,(u,v)\,\frac{\tau_K}{1+(\omega\tau_K)^2} \rightarrow \frac{\tau_0}{1+(\omega\tau_0)^2}$. In that limit the authors of mr-2021-35 maintain that $(1-S^2)\,P_2(u\cdot v) \rightarrow S_{uv}^2$. Thus, mr-2021-35 predicts the following form for $J_{u,v}(\omega)$ in the limit of isotropic global motion:

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

where $\tau_e^{-1} = \tau_{int}^{-1} + 6D_\perp$ and $S_{uv}^2 = S^2\,P_2(u\cdot v)$. Yet, in this limit one should have:[2]

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

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

(c) The purpose of using the cross-correlation-related expression $\sum_{K=0,1,2} A_K\,(u,v)\,\frac{\tau_K}{1+(\omega\tau_K)^2}$ (eq A.15 of ref 2), instead of the auto-correlation-related expression $\sum_{K=0,1,2} A_K\,(u)\,\frac{\tau_K}{1+(\omega\tau_K)^2}$ (eq A.14 of ref 2) is to allow the effective polar

angle, α, in $P_2(u \cdot v) = P_2(\cos \alpha)$, to be relatively large, as it is for the C^{α}–C' spin system (fourth bullet item in mr-2021-35-AC2-supplement). Let us examine this matter. The section after eq A.15 of ref 2 states the following:

> in general $\langle P_2(\mu_p(0)\cdot\mu_p(t))\rangle \neq \langle P_2(\mu_q(0)\cdot\mu_q(t))\rangle \neq \langle P_2(\mu_p(0)\cdot\mu_q(t))\rangle P_2(\cos\theta_{pq})$. Thus, the approach used in the text to treat anisotropic overall motion is far from rigorous, although it is expected to be a reasonable approximation when θ_{pq} is small.

The first two TCFs in this citation yield by Fourier-Laplace transformation the spectral densities $J_{u,u}(\omega)$ and $J_{v,v}(\omega)$ (in the notation of mr-2021-35). "The approach used in the text" refers to the spectral density:

$$J_{u,v}(\omega) = P_2(u \cdot v)\left[S^2 \sum_{K=0,1,2} A_K(u) \frac{\tau_K}{1+(\omega\tau_K)^2} + (1 - S^2)\frac{\tau_e}{1+(\omega\tau_e)^2}\right] \qquad (4)$$

The polar angle, α, has to be small enough so that the auto-correlated spectral densities, $J_{u,u}(\omega)$ and $J_{v,v}(\omega)$, are within a good approximation the same. Otherwise the expression in the square brackets of eq 3, which represents the MF spectral density where $J(\omega) = J_{u,u}(\omega) = J_{v,v}(\omega)$, may not be used. Yet, eq 3 is the limit of eq 1 for isotropic global diffusion. For the same reason – inherent equality between $J_{u,u}(\omega)$ and $J_{v,v}(\omega)$ – α has to be small in eq 1. As delineated in my original review report, additional geometric simplifications are inherent in eq 3; they are also inherent in eq 1.

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.

- Eq. (2) (mr-2021-35) is very general. We cannot follow how it might be invalid. In fact, the study of (Tjandra et al. 1996) highlighted as counter-argument features this very expression already in Eq. (2) as well as in the appendix Eq. (A.1).
- The same holds true for the objected expression (3) (mr-2021-35). Eq. (3) is a very general form of a TCF. It only implies that the decay can be modeled as a superposition of exponential decays. All commonly employed analytical models and even MD-extracted TCFs adhere to this general shape. We believe sufficient references have been provided.
- From (2) and (3) follows (4) (mr-2021-35), so we must disagree with the objections raised. With the angle between **u** and **v** fixed, the same expression is also found in (Tjandra et al. 1996) between (A.1) and (A.2).

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.

- 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 sequential NOEs, this model has been invoked to rationalize unexpected variations in α-

Synuclein (Mantsyzov et al. 2014, p. 1281-1282). However, as $H^{\alpha}H^{N}$ distances vary with φ and ψ, the observed effects were ultimately considered to be dominated by distance variations (p. 1286). $C'C^{\alpha}$ CCR would not suffer from this ambiguity, which is why we propose it as an alternative.

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

- We do not understand in what way Eqs. (6) and (7) (mr-2021-35) are confusing. Again, they are taken from the highlighted study of (Tjandra et al. 1996), see Appendix (A.14). The expression suggested by referee #2 makes use of the auto-correlated expression (A.15). We already commented on the possibility to approximate the entire cross-correlated TCF by an auto-correlated TCF on page 6. As the angle between $C'C^{\alpha}$ and σ_{yy} is rather large, we prefer to model $C_{tumb}(t)$ according to (A.14). Referenced in the paper, a different representation has 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.

- 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 sequential NOEs, this model has been invoked to rationalize unexpected variations in α-

Synuclein (Mantsyzov et al. 2014, p. 1281-1282). However, as $H^{\alpha}H^{N}$ distances vary with φ and ψ, the observed effects were ultimately considered to be dominated by distance variations (p. 1286). $C'C^{\alpha}$ CCR would not suffer from this ambiguity, which is why we propose it as an alternative.

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

- In addition, to **assess** the effect of isotropic local motions, we simply introduce an additional exponential decay / Lorentzian. As we said in the manuscript "While the fast isotropic motions could be modeled in more detail to better fit the shape of the TCF using e.g. the extended MF approach(Clore et al., 1990) or correlation time distributions(Hsu et al., 2018), we only intend to divide J(0), i.e. the TCF's enclosed area, into contributions with and without orientational biases." (page 6). Eq. (10) (mr-2021-35) is a rough MF-like approximation, $(1\text{-}S^2)\tau_3$ is simply the contribution to J(0) attributed to isotropic motions. While in principle arbitrary how this contribution is denoted, S^2 and τ_{int} tend to provide a better "feel" for many. Including additional and/or differently termed isotropic terms would not change the behavior of Q, only their cumulative size is relevant.

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.

Our approximation can be justified from various angles which we have sketched in the paper. While one can argue about the physical meaning of MF-type models, two different approaches connecting τ_{int} with τ_0 τ_1 τ_2 were highlighted. We agree that the word "coupling" is a poor choice. We were referring to how the factorization (which generally implies **no** dynamic coupling) $C_{tumb}(t)C_{int}(t)$ is handled. It should be noted that already this product form is not strictly applicable in case of anisotropic tumbling. (Kroenke et al. 1998) keep $C_{tumb}(t)$ anisotropic, Eq. (1), which yields a τ_3, τ_4 and τ_5 and retains orientational biases even with S^2=0. We follow (Barbato et al. 1992), Eqs. (6a) and (6b) / (Tjandra et al. 1995), sec. Theory, who approximate τ_3 assuming an effective isotropic $C_{tumb}(t)$, see below. $C_{int}(t)$ decays from $P_2(\mathbf{u}\cdot\mathbf{v})$ towards S^2_{uv} which for the approximated isotropic tumbling can be expressed as $S^2_{uv} = S^2P_2(\mathbf{u}\cdot\mathbf{v})$ (Ghose et al., 1998), Eq. (19), Appendix, or (Fischer et al. 1997), Eq. (29), which yields the "second term of eq 3".

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.

The MF approach of (Halle 2009), which does not assume the factorization of $C_{tumb}(t)C_{int}(t)$, was highlighted as well. The auto-correlated expression for anisotropic diffusion is found in Eq. (2.40). Treatment of cross-correlations are described in section D. Replacing 1 in Eq. (2.40) with $\kappa_{uv} = P_2(\mathbf{u}\cdot\mathbf{v})$, Eq. (2.59), and again approximating $S^2_{uv} = S^2\kappa_{uv} = S^2P_2(\mathbf{u}\cdot\mathbf{v})$, the expressions of (Tjandra et al. 1996), Eqs. (3), (4) and (6) are obtained. These correspond to Halle's Eq. (2.64) for isotropic internal mobility which relates the cross-correlated TCF to the auto-correlated TCF as suggested by referee #2. The chosen exponential form of $C_{int}(t)$ and the choice of τ_{eff} can be motivated as above. If the angular dependencies of $C_{tumb}(t)$ are encoded by Eqs. (6) and (7) (mr-2021-35) instead of $S^2_{uv} = S^2P_2(\mathbf{u}\cdot\mathbf{v})$, Eq. (10) (mr-2021-35) is obtained. The expression suggested by (Ghose et al., 1998), Eq. (7), is very similar but lacks the prefactor $P_2(\mathbf{u}\cdot\mathbf{v})$ for the internal TCF, which is problematic for large angles (e.g. between C'C^{α} and σ_{yy}). Regarding the necessity of various assumptions (including frame transformation properties) as well as the general validity of different MF approaches, we find Halle's remarks in VI.A worth highlighting.

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.

We feel Eq. (10) (mr-2021-35) adheres closely to conventional descriptions of MF-adjusted diffusion anisotropy. Again, we are not claiming this is how an IDP will realistically behave. Rather, what signature would the simplified image of anisotropic tumbling imply? And to what extent could we still detect it if we include faster isotropic motions in a simplified manner?

One cannot attain objectives with inadequate tools.

- "Why should the effective correlation time for global motion be equal to τ_2^{-1}?" It appears referee #2 is mistaken, $\tau_{eff} = (4D_{\perp} + 2D_{\parallel})^{-1} \neq (4D_{\parallel} + 2D_{\perp})^{-1} = \tau_2$. As we referenced

(Barbato et al. 1992, Tjandra et al. 1995), it is calculated from the trace of the diffusion tensor

$$\tau_{eff} = 6D^{-1} = 6\,{}^{1}\!/_{3}\,(D_x + D_y + D_z)^{-1} = 6\,{}^{1}\!/_{3}\,(D_{\perp} + D_{\perp} + D_{\parallel})^{-1} = (4D_{\perp} + 2D_{\parallel})^{-1}$$

My apologies for this oversight (see above).

General matters.

(2) A cursory survey of the NMR-based studies of IDPs cited in mr-2021-35 shows that methods examining local features point to a random-coil situation (e.g., see Mantsyzov et al. **2014**). CCR in $^{15}N–^{1}H$ and $^{13}C'–^{13}C^{\alpha}$ is a local feature. Obtaining information on long-range "order", in particular "diffusion anisotropy", requires empirical spectral densities comprising statistical elements, to be used in combination with coil libraries and molecular dynamics simulations (e.g., see Mantsyzov et al. **2014**). These elements are absent in mr-2021-35 scheme.

> **(2)** As stated before, the concept of anisotropic tumbling of α-helical and chain-like elements has been invoked in that very same paper (Mantsyzov et al 2014), pages 1281-1282. It was speculated that the local orientation of the spin pairs with respect to the $C^{\alpha}C^{\alpha}$ vector (in part) explains the variations of intraresidual and sequential $^{1}H^{\alpha i}H^{N}$ NOEs.

This is a description of matters addressed in ref 1; it is not a response to comment 2. Please see above for my suggestion in this regard.

(3) It is indicated that anisotropic "segmental" motion is targeted. How are these "segments" defined? In other words, how is the second-rank diffusion tensor, ***D***, defined?

> **(3)** Following (Mantsyzov et al 2014), we assume that the peptide plane is embedded within the same diffusion tensor. Its unique axis is assumed to lie in the peptide plane such that the sketched edge cases (parallel/perpendicular) are covered. The details are described in the Methods section.

Please see above for related comments/discussion.

(4) It is pointed out that NMR relaxation analysis methods applicable to folded proteins are not applicable to IDPs. The model-free (MF) spectral density, a variant of which is suggested here, refers to protein and probe as rigid bodies moving in a statistically independent (decoupled) manner. In IDPs the protein is not rigid and its motion is not decoupled from the motion of the probe. The spectral density suggested here does not reflect these features; rather, it is similar in character to the MF spectral density.

> **(4)** As we mentioned before, we do not expect Eq. (10) (mr-2021-35) to apply in any strict sense. It is indeed the spectral density for MF-like anisotropic tumbling of a (sufficiently) rigid symmetric top. That being said, the general form of Eq. (10), i.e. a weighted sum of Lorentzians, can be expected to describe the TCFs of virtually any protein system in isotropic solution.

I addressed these issued above. Only an infinite sum of Lorentzians describes any system involved in rotational reorientation. Finite sums will be appropriate if they are solutions of physical-relevant models.

(5) In the context of item (2) – please note that the "dynamics detectors" method (Smith et al. *Angew. Chem. Int. Ed.* **2017** *56*. 13590), shown to actually surpass MF, comprises statistical elements. Recently it was applied to proteins in solution (Smith et al. *JCP* **2019**, *151*, 034102). The authors might want to check applicability to IDPs.

(5) We agree that fitting experimental relaxation parameters with only few Lorentzians has its limitations. The number of parameters and consequential statistical uncertainties are problems in their own right. In fact, Crawley and Palmer address this issue in this Festschrift (mr-2021-28). In our study, we have referenced different possibilities ranging from correlation time distributions to spectral density visualizations. Again, we are interested in detecting effects of anisotropic dynamics in J(0). We do not intend nor suggest to fit experimental relaxation parameters of IDPs using Eq. (10) (mr-2021-35).

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

References

1. Mantsyzov, A. B.; Maltsev, A. S.; Ying, J.; Shen, Y.; Hummer, G.; Bax, A. A Maximum Entropy Approach to the Study of Residue-Specific Backbone Angle Distributions in α-Synuclein, an Intrinsically Disordered Protein. *Protein Science* **2014**, *23*, 1275-1290.

2. Tjandra, N.; Szabo, A.; Bax, A. Protein Backbone Dynamics and ^{15}N Chemical Shift Anisotropy from Quantitative Measurement of Relaxation Interference Effects. *J. Am. Chem. Soc*. **1996**, *118*, 6986-6991.

3. Lipari, G.; Szabo, A. Model-Free Approach to the Interpretation of Nuclear Magnetic Resonance Relaxation in Macromolecules. 1. Theory and Range of Validity. *J. Am. Chem. Soc*. **1982**, *104*, 4546-4559.

4. Halle. B. The Physical Basis of Model-Free Analysis of NMR Relaxation Data from Proteins and Complex Fluids. *J. Chem. Phys*. **2009**, *131*, 224507-22.

5. Ghose, R.; Huang, K.; Prestegard, J. H. Measurement of Cross Correlation between Dipolar Coupling and Chemical Shift Anisotropy in the Spin Relaxation of ^{13}C, ^{15}N-Labeled Proteins. *J. Magn. Res*. **1998**, *135*, 487-499.

Summary: Please provide a cross-correlated spectral density which forgoes the deficiencies pointed out, and relate to the comments made.