The authors of this manuscript mr-2021-35 suggest a strategy for detecting anisotropic diffusion, $D_\perp \neq D_\parallel$ (where $\boldsymbol{D}$ denotes a second-rank diffusion tensor), in intrinsically disordered proteins (IPDs), on the basis or NMR cross-correlated relaxation (CCR). The spin systems considered include $^{15}\text{N}–^1\text{H}$ and $^{13}\text{C'}–^{13}\text{C}^\alpha$. It was shown previously for $^{15}\text{N}–^1\text{H}$ that the transverse and longitudinal CCR rate constants can be combined linearly so as to yield an expression which depends solely on the measurable spectral density, $J^{CD}(\omega)$, for $\omega = 0$ ("C" stands for the ^{15}N CSA interaction and "D" for the $^{15}\text{N}–^1\text{H}$ dipolar interaction). This strategy is adopted here for the $^{13}\text{C'}–^{13}\text{C}^\alpha$ spin system (with "C" standing for the $^{13}\text{C'}$ CSA interaction and "D" for the $^{13}\text{C'}–^{13}\text{C}^\alpha$ dipolar interaction). An improved experimental method for measuring the longitudinal CCR rate constant for $^{13}\text{C'}–^{13}\text{C}^\alpha$ is reported. $J^{CD}(0)$ data are acquired experimentally for both spin systems. On the theoretical side a new form for $J^{CD}(\omega)$, which should enable detecting $D_\perp \neq D_\parallel$ when $\omega = 0$, is suggested. Detection sensitivity is illustrated. My comments refer to several matters of general character, and to the form of the $J^{CD}(\omega)$ function.

General matters. **(1)** This study is totally predictive in nature. No validation, and/or examples illustrating actual applicability are provided. **(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 $^{15}\text{N}–^1\text{H}$ and $^{13}\text{C'}–^{13}\text{C}^\alpha$ 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. **(3)** It is indicated that anisotropic "segmental" motion is

targeted. How are these "segments" defined? In other word, how is the second-rank diffusion tensor, D, defined? **(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. **(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.

$J^{CD}(\omega)$. Let us focus on $^{15}N-^{1}H$ spin system as paradigm. The essence of the following is equally applicable to the spin system $^{13}C'-^{13}C^{\alpha}$. Within a very good approximation the $^{15}N-^{1}H$ dipolar/^{15}N CSA cross-correlated spectral density, $J^{CD}(\omega)$, is given for globular proteins by (Tjandra et al. *JACS* **1996**, *118*, 6986):

$$J^{CD}(\omega) \cong P_2(\cos\theta)\, J^{DD}(\omega) = P_2(\cos\theta)\, j_0(\omega) \tag{1}$$

where θ denotes the angle between the principal axes of the axial $^{15}N-^{1}H$ dipolar and ^{15}N CSA tensors. $J^{DD}(\omega)$ is the measurable spectral density for auto-correlated dipolar relaxation. $j_0(\omega)$ is the $K = 0$ component of the solution, $j_K(\omega)$, $K = 0, 1, 2$, of the dynamic model considered. *For wobble-in-a-cone $j_0(\omega)$ is given by the MF spectral density* (Lipari & Szabo *JCP* **1981**, *75*, 2971).

The point I wish to make is as follows. The physical picture underlying NMR relaxation is inherent in the $j_K(\omega)$, $K = 0, 1, 2$, functions, which are not measurable. To render them measurable one has to carry out appropriate frame transformations. The equality in eq 1 is due to the fact that the model-related local ordering frame, M, where the $j_K(\omega)$ functions are defined, and the NMR-related D frame, are taken the same in MF. The approximate equality in eq 1 is due to the fact that the C frame and the M = D frame are not the same but θ is small (Tjandra et al. *JACS* **1996**, *118*, 6986). Hence, only the $K = 0$ component, $P_2(\cos\theta)$, in the M = D to C frame transformation survives.

Thus, J^{DD}, J^{CC} and J^{CD} are geometric implementations of $j_K(\omega)$, $K = 0, 1, 2$. Equation (2) of mr-2021-35 represents time-dependent cross-correlation between the (axial) D and C frames. As shown above, the C frame is obtained from the D (= M) frame by a frame transformation based on time-independent Euler angles. This post-solution frame transformation can also be found in a different but related case in Szabo *JCP* **1980**, *72*, 4620. This invalidates eq 2.

Equations 2–4 do not comply with the theory of moments, which underlies the MF time correlation function utilized here as basis. Equation 6 represents the frame transformation from the $^{15}N-^{1}H$ dipolar frame (u) to the global diffusion frame (Tjandra et al. *JACS* **1995**, *117*, 12562). One should have:

$$\sum_{K=0,1,2} A_K(u) \frac{\tau_K}{1 + (\omega\tau_K)^2} \qquad (2)$$

The index, v, in eq 6 $[(A_K(u,v)]$ is not comprehensible in this context. Equation 7 is very confusing. The coefficients $A_K(u)$ are in actual fact time-independent trigonometric functions. Yet, the quantities $A_K(u,v)$ in eq 7 of mr-2021-35 feature as coordinates "(θ, φ), which denote the

polar angles in the tumbling frame"; this implies time-dependence of angles alien to the essence of $A_K(u)$.

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

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

where $\tau_3^{-1} = \tau_{int}^{-1} + 4 D_\perp + 2 D_\parallel$. The correlation time, τ_{int}, is considered to be the average correlation time for internal motion. The correlation time $\tau_{eff} = (4 D_\perp + 2 D_\parallel)^{-1}$ is considered to be the effective correlation time for global tumbling. The two principal values, $D_\perp$ and $D_\parallel$, of the second-rank diffusion tensor correspond to the three eigenvalues of the symmetric top, given by $\tau_K^{-1} = 6 D_\perp + K^2(D_\parallel - D_\perp)$, $K = 0, 1, 2$. This yields $\tau_0^{-1} = 6 D_\perp$, $\tau_1^{-1} = D_\parallel + 5 D_\perp$, and $\tau_2^{-1} = 4D_\parallel + 2 D_\perp$. Why should the effective correlation time for global motion be equal to τ_2^{-1}? In what sense does the expression $\tau_{int}^{-1} + 4 D_\perp + 2 D_\parallel$ represent – as indicated – dynamical coupling, which means concerted time-evolution of the global and local motional degrees of freedom? What physical framework could possibly justify the second term of eq 3?