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Inhibitor-directed spin labelling – a high precision and minimally invasive technique to study the conformation of proteins in solution

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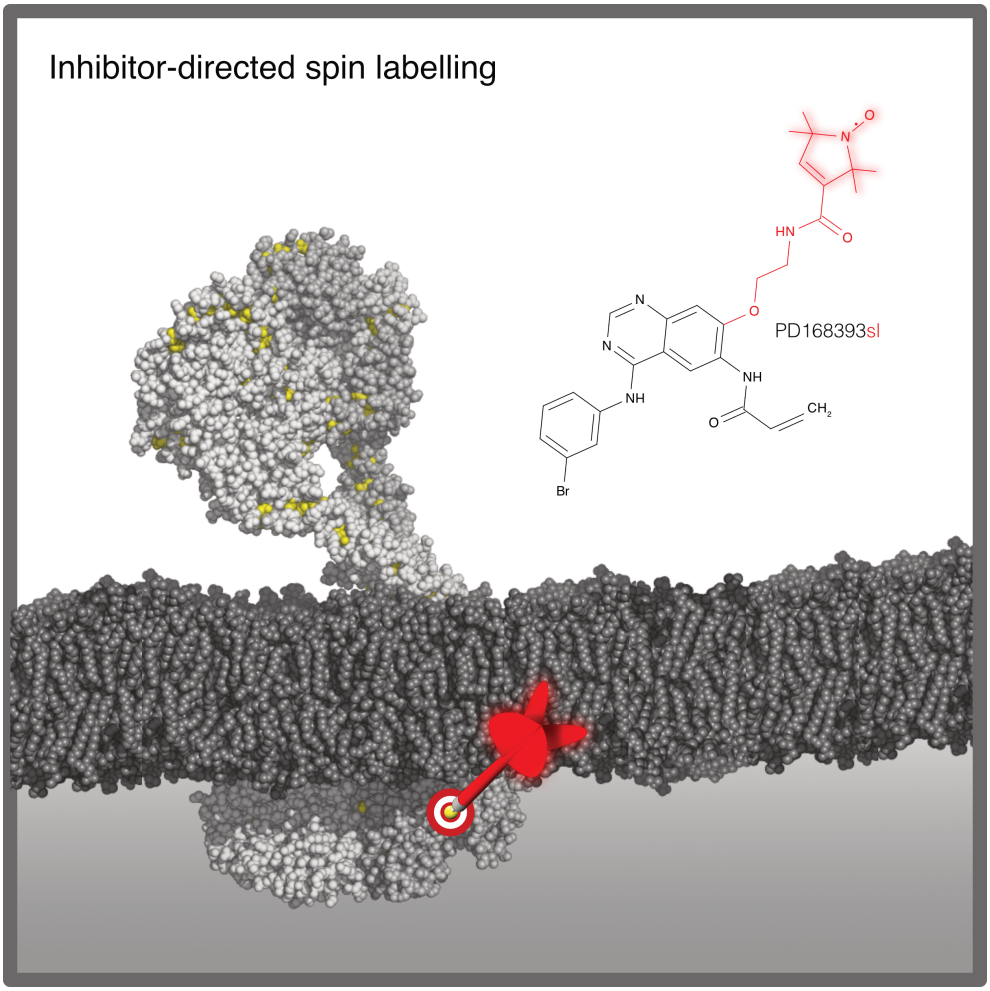

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Abstract: Pulsed electron-electron double resonance spectroscopy (known as PELDOR or DEER) has recently become a very popular tool in structural biology. The technique can be used to accurately measure distances within macromolecules or macromolecular complexes, and has become a standard method to validate structural models and to study the conformational flexibility of macromolecules. It can be applied in solution, in lipid environments or even in cells. Because most biological macromolecules are diamagnetic, they are normally invisible for PELDOR spectroscopy. To render a particular target molecule accessible for PELDOR, it can be engineered to contain only one or two surface-exposed cysteine residues, which can be efficiently spin-labelled using thiol-reactive nitroxide compounds. This method has been coined "site-directed spin labelling" (SDSL) and is normally straight-forward. But, SDSL can be very challenging for proteins with many native cysteines - or even a single functionally or structurally important cysteine residue. For such cases, alternative spin labelling techniques are needed. Here we describe the concept of "inhibitor-directed spin labelling" (IDSL) as an approach to spin label suitable cysteine-rich proteins in a site-directed and highly specific manner by employing bespoke spin-labelled inhibitors. Advantages and disadvantages of IDSL are discussed.

Since the determination of the first macromolecular structures in the 1950s, structural biology has made remarkable progress and continues to provide intriguing glimpses at the atomistic details of life. For many years, macromolecular crystallography and nuclear magnetic resonance spectroscopy (NMR) have very successfully led the way towards unravelling the structure and function of ever more complex biological machineries, as witnessed by the currently more than 130.000 structural models in the protein database (PDB, www.rcsb.org). In recent years, the field of structural biology is rapidly changing. New developments in cryo-electron microscopy have propelled this technique to the forefront of the field and this will have a profound effect on the future of structural biology [1]. The recent years have also witnessed the maturation of several other techniques such as single molecule Förster resonance energy transfer spectroscopy (smFRET) [2,3] and small-angle X-ray scattering (SAXS) [4], which have been established as important methods in structural biology. This growing box of tools has led to the concept of "integrative structural biology", where combinations of suitable methods are used to tackle complex structural problems that would be difficult or impossible to solve by one technique alone [5]. One of the relatively new additions to the toolbox is pulsed electron-electron double resonance spectroscopy (PELDOR, also known as DEER) [6,7]. PELDOR is an electron paramagnetic resonance (EPR) spectroscopy technique that can be used to precisely measure long distances (15-160 Å[8,9]) within a macromolecule or macromolecular complex. The technique can be applied to macromolecules of virtually any size and it works in frozen solution, lipid environments, native membranes or even in cells [10,11]. In numerous instances, PELDOR has been used to test, whether a particular crystallographic model correctly reflects the solution state of a macromolecule or to follow conformational changes in response to the binding of a cofactor or substrate (e.g. [12-17]). In combination with NMR spectroscopy, the introduction of long-distance constraints from PELDOR opens new avenues at studying large macromolecular complexes [18].

Most macromolecules are diamagnetic and therefore invisible to EPR spectroscopy. Therefore, to make use of PELDOR spectroscopy, spin labels such as the commonly used MTSSL (*S*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrol-3-yl)methyl methanesulfonothioate) have to be attached to the target molecule [19] (Figure 1A). The distance between the introduced spin labels can then be measured and translated into structural information with the help of various *in silico* approaches [20,21].

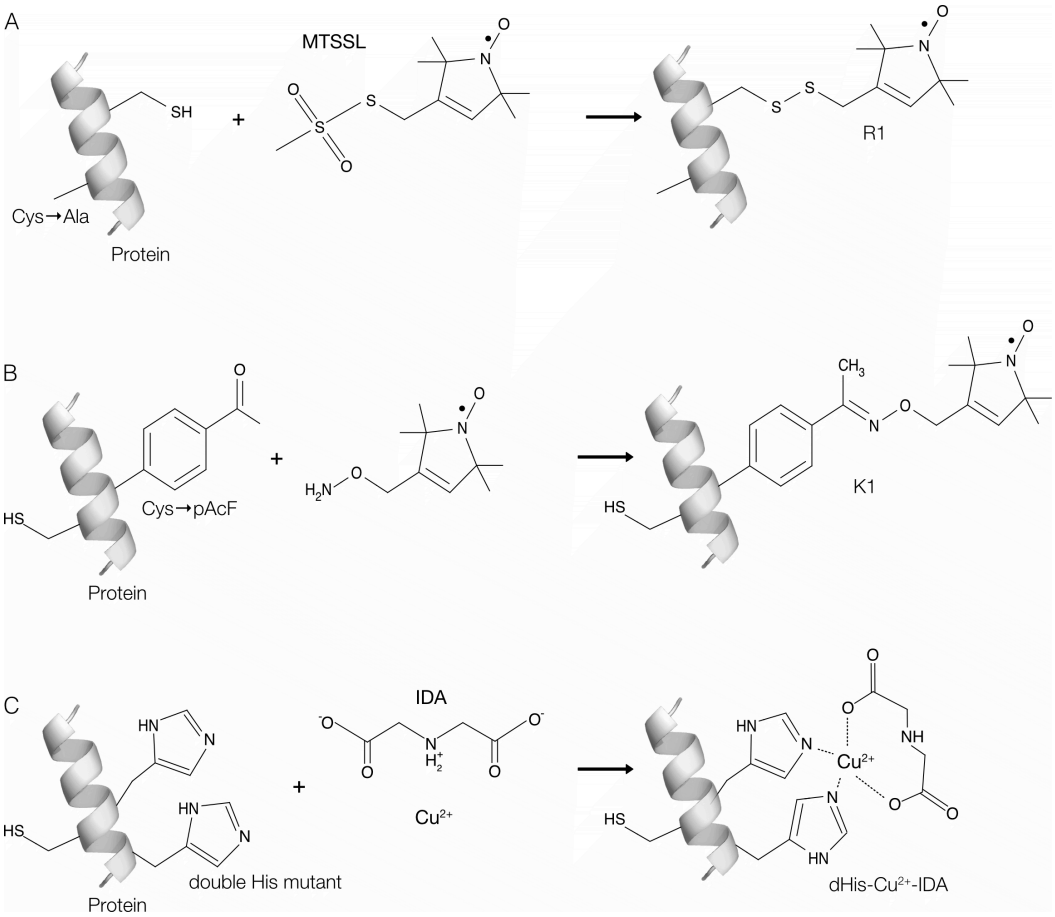

Figure 1: Examples of different spin labelling techniques. A) "Traditional" site directed spin labelling with MTSSL. Any solvent accessible cysteines have to be replaced by alanine or serine in order to introduce the spin label site-directedly [22]. **B)** Spin labelling by introduction of an unnatural amino acid such as para-acetylphenylalanine (pAcF) can be performed without removing the native cysteine residues [23]**. C)** The introduction of metal ions as spin centers can be achieved by engineering two closely spaced His residues. Together with Cu^{2+} ions and iminodiacetic acid (IDA), a stable spin center is formed [24].

The standard procedure to attach spin labels to a target molecule is, to introduce a surface cysteine residue and to react it with the thiol-reactive MTSSL label. If only a few native cysteines are present in the target, they can be replaced by alanine or serine, often without disturbing the structure or the biological activity of the macromolecule. Still, for some proteins, this is not a viable option, because the native cysteines are either functionally relevant or because there are simply too many cysteine residues in the protein. A possible solution for such cases is the use of unnatural amino acids, for instance para-acetylphenylalanine or propargyl-lysine. These residues can be introduced using the techniques established by the Schultz laboratory [25] and then be specifically labelled e.g. via click-chemistry or any other type of reaction that is compatible with aqueous buffers and the

particular requirements of the target protein [23,26,27] (Figure 1B). However, sometimes even a single mutation is not tolerated or the unnatural amino acid is not efficiently incorporated, leading to lower expression yields. In such instances, the introduction of metal centers such as Cu^{2+} into the target protein might be an option. This can be achieved by introducing two closely spaced histidine residues. Together with iminodiacetic acid (IDA), the histidines can coordinate Cu^{2+} ions, leading to a rigid but structurally rather demanding spin label (Figure 1C) [24].

A good example for the difficulties that can be encountered with spin labelling EPR is our previous study on the epidermal growth factor receptor (EGFR). The EGFR is a large single-pass transmembrane protein with 1186 amino acid residues (Figure 2A). The extracellular domain (ECD) of the protein acts as a receptor for the epidermal growth factor family of ligands. Once activated by ligand binding, the intracellular kinase domains of two receptors are thought to form an asymmetric dimer leading to trans-autophosphorylation of their C-terminal tails [28]. Inside the cell, this signal is then propagated and amplified by effector proteins and ultimately promotes cell proliferation, inhibition of apoptosis and various other processes [29]. Due to this involvement in cell cycle control, many mutants of the EGFR are oncogenic, rendering the protein a very important anti-cancer drug target. Structurally, the protein is well characterized, with several high-resolution structures of the intracellular kinase domain and separate structures of the extracellular domains [30]. The function of EGFR is critically dependent on conformational changes, and it is therefore of high interest to study its structure in solution, without the rigid corset of the crystal lattice. Efforts in this direction have been made with molecular dynamics simulations [31], but, direct experimental evidence of the MD-based full-length structures was lacking. We therefore embarked on a study of the conformation of the full-length EGFR or its intracellular domains (ICDs) in solution using PELDOR spectroscopy. The results of these efforts have been previously published [32]. Here, we describe the difficulties that were encountered along the way and how the concept of inhibitor-directed spin labelling (IDSL) was conceived, which ultimately allowed us to solve this difficult structural problem.

During the planning stages of our study, we analyzed available structures of EGFR dimers [31] to identify suitable labelling positions. Due to the dimeric structure, the introduction of only one spin label suffices to perform PELDOR distance measurements.

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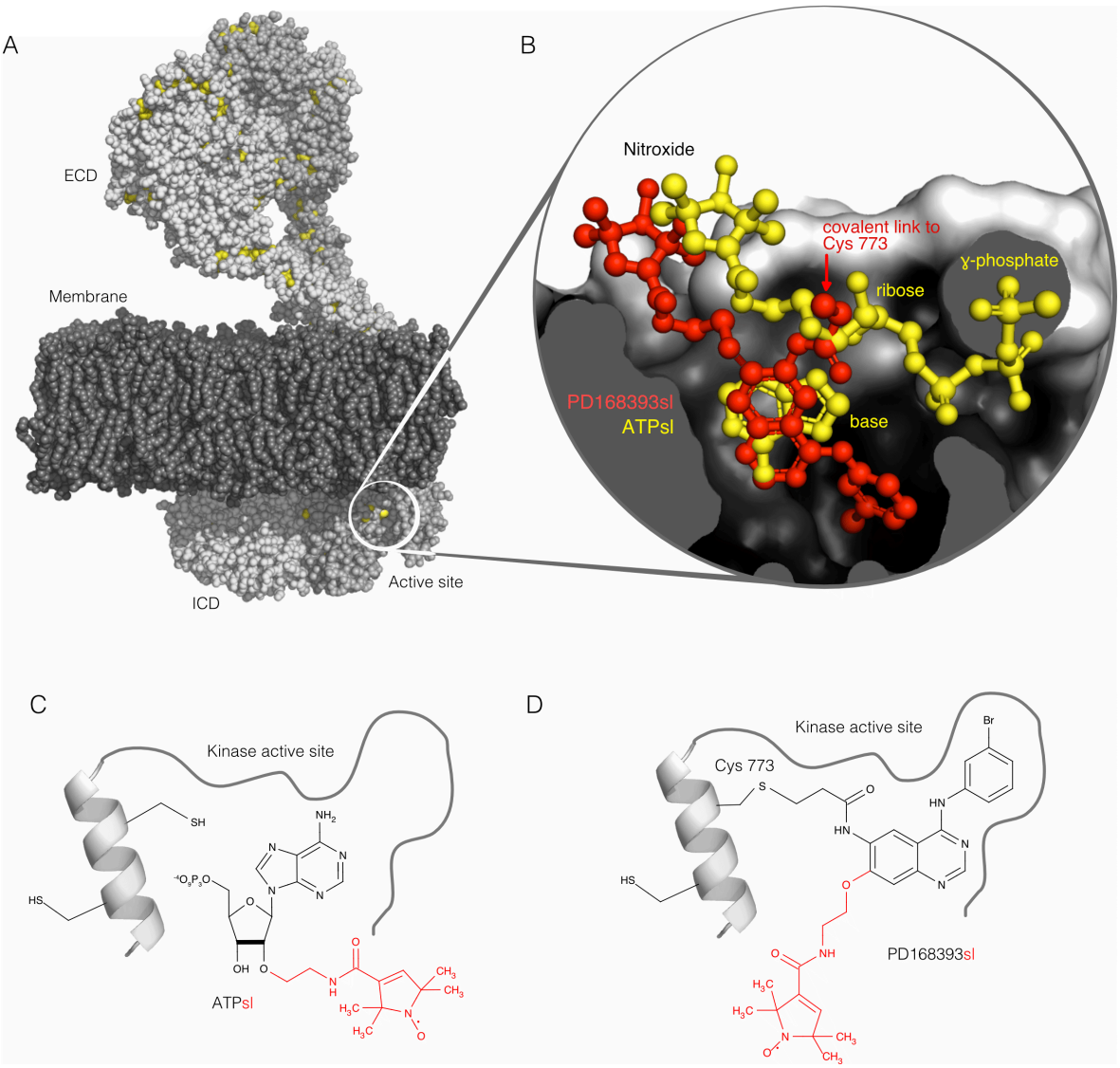

Figure 2: Comparison of inhibitor-based spin labelling (IDSL) and cofactor-based spin labelling of the EGFR. A) A space filling model of the EGFR in its active state [31]. The membrane is coloured dark grey and the two chains of the EGFR dimer in lighter shades of grey. Cysteine residues are coloured yellow. The cysteine residue in the kinase active site is marked with a white circle. The extracellular domain (ECD) and intracellular domain (ICD) are labelled. **B)** Close-up view of the EGFR kinase active site. The protein is shown as a grey surface. ATPsl (yellow) and PD168393sl (red) were modelled with mtsslWizard based on crystal structures 2GS6 [33] and 4LRM [34], respectively, and are shown as ball-and-sticks. The structural features of ATP are labelled for orientation. **C, D)** Chemical formula of the ATPsl (C) and PD168393sl (D) spin labels. The modification is highlighted in red. The active site pocket of the EGFR is symbolised by a grey curve.

Despite the large size of EGFR, only three sites (R662, S744 and D806) fulfilled all our criteria for the selected residues: low conservation, high surface accessibility and an expected inter-spin distance within the optimal PELDOR range of 20-80 Å (longer distances can be measured but require fully deuterated proteins [9,35]). However, the EGFR has dozens of native cysteines and the ICD alone contains nine cysteine residues, six of which are located in the kinase domain and the remaining three in the C-terminal tail (Figure 2A, yellow spheres). The four solvent-exposed cysteines of the ICD are likely accessible for MTSSL and had to be replaced by serine or alanine. Also, the C-terminal tail was removed from the protein. The mutant proteins could be produced in SF9 cells with sufficient yields and were successfully spin labelled with MTSSL. Unfortunately, in contrast to the C-terminally truncated - but otherwise wild-type - ICD, the mutants revealed a severely attenuated autophosphorylation activity.

We reasoned that PELDOR data from such inactive mutants would not allow us to draw any valid conclusions concerning the function of the wild-type protein. As already mentioned, it is in such cases a good alternative to leave the native cysteines untouched and to instead introduce an unnatural amino acid such as para-acetylphenylalanine (see above, Fig 1B) [23]. However, because the EGFR kinase domain proved recalcitrant to even minor changes (Cys→Ser or Cys→Ala), we were reluctant to introduce any mutations into the protein. Also, at least in our experience, the commonly used labelling procedures for unnatural amino acids (involving low pH or Cu^{2+} ions) are not tolerated very well by unstable proteins such as EGFR. Note that solutions to improve these problems are a matter of current research [26,27].

In previous studies on unrelated proteins, chemically modified substrates were used as spin labels for PELDOR [11,36]. Inspired by this work, we decided to follow a similar approach in our study. Since EGFR is its own substrate, the approach was not directly applicable. However, ATP, the cofactor in the phosphorylation reaction, is an alternative option to introduce the spin label[37,38]. We therefore synthesized a spin labelled variant of ATP by fusing the commercially available 1-oxyl-2,2,5,5-tetramethylpyrroline-3-carboxylate N-hydroxysuccinimide ester spin label (also known as oxyl-1-NHS) to the 2'-OH position of the ATP ribose moiety via a flexible C2 linker (Figure 2B). Details of the synthesis and the accompanying analytics are shown in the supplementary information. Modelling this molecule (ATPsl) into the crystal structure of the EGFR kinase domain confirmed that it does fit into the ATP binding pocket with the nitroxide group facing the solvent (Figure 2B, yellow). Also, autophosphorylation assays with EGFR and ATPsl demonstrated that the modified cofactor is still accepted by the EGFR (Figure 3A).

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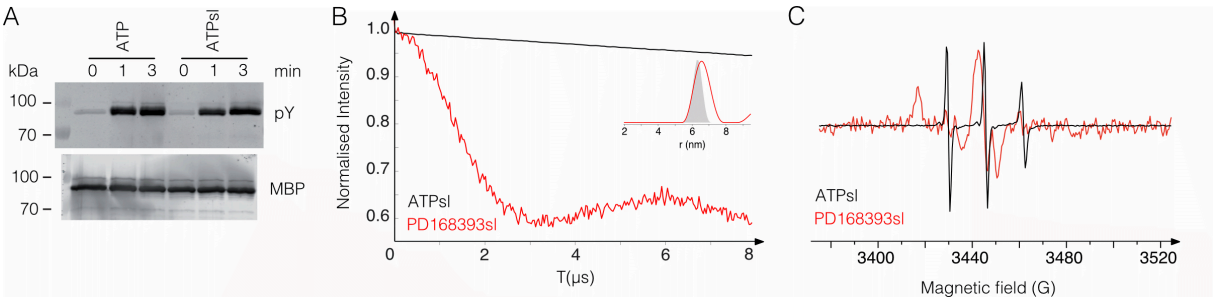

Figure 3: Inhibitor-based spin labelling (IDSL) vs cofactor-based spin labelling of the EGFR. A) EGFR autophosphorylation assay using ATP or ATPsl as a cofactor. Phosphorylated MBP-EGFR-ICD998 (pY) was visualized by an anti-phosphotyrosine antibody and total amount of MBP-EGFR-ICD998 (MBP) by anti-MBP antibody. **B)** PELDOR time traces (not background corrected) obtained from 1:1 mixtures of ATPsl or PD168393sl with MBP-EGFR-ICD998 (black and red lines, respectively). The inset shows the distance distribution that was derived from the red time trace using DeerAnalysis [39]. The grey shade represents the inter-spin distance as predicted by mtsslWizard [40]. **C)** Room temperature X-band cw-EPR spectrum of a 1:1 mixture of EGFR and ATPsl (black trace) and PD168393sl (red trace). The actual PELDOR samples were used for the measurement. See [32] for a discussion of the PD168393sl line shape.

Purified EGFR was then incubated with an equimolar amount of ATPsl and analyzed by PELDOR. Disappointingly, as can be seen in Figure 3B (black trace), the PELDOR time trace showed no visible modulation. At the same time, the room temperature X-band cw-EPR spectrum of the PELDOR sample revealed three sharp lines, which are indicative of an unbound nitroxide spin label [41] (Figure 3C). The K_M of ATP for EGFR was previously determined to be ~5 µM [42]. Assuming that the K_d is in the same range and at total concentrations of 30 µM for both EGFR and ATPsl in the PELDOR sample, around 20 µM EGFR-ATPsl complex were expected to be observed. But, apparently, the introduction of the spin marker at the 2'-OH position lowers the affinity of the cofactor, so that high excesses of ATPsl would be needed to promote complex formation. This would inevitably lead to a large amount of unbound ATPsl, defeating the purpose of the experiment.

In all likelihood, this will be a problem with many natural ligands, for which affinities are generally not very high and often not many possibilities for the attachment of spin labels exist. At this point we were faced with two options: 1) improving the binding affinity of ATPsl, for example by trying different attachment points for the nitroxide group (the 3'-OH of the ribose moiety would be an obvious choice; Figure 2C), or 2) discarding the cofactor approach. Although option 1) might have eventually been successful, we decided against it due to an additional argument: Most cofactors are not specific for a given enzyme but are

used by a whole family of enzymes or even by many different enzyme classes. This is of course especially true for ATP but also for other common cofactors such as NAD.

At this point, we had the idea that both the affinity- and specificity problems could be circumvented by spin labelling specific high-affinity inhibitors instead of substrates or cofactors: A specific high-affinity inhibitor can be utilized to place the spin label at a defined site in the otherwise unmodified protein, even in the presence of other proteins. In contrast to substrates or cofactors, many inhibitors have dissociation constants in the low nanomolar range or even below. Therefore, the unbound fraction can be neglected at the micromolar protein concentrations that are typically used in PELDOR experiments. In addition, an increasing number of covalent inhibitors exist, eliminating the potential problem of dissociation completely [43]. The EGFR inhibitor PD168393 is such a covalent inhibitor [44]. It has an α,β-unsaturated carbonyl group, which covalently attaches to Cys 773 in the ATP-binding cleft of EGFR, utilizing a Michael addition mechanism. Importantly, the replacement or protection of other cysteine residues in the protein is unnecessary as the probe is targeted specifically to the cysteine residue in the binding site of the inhibitor. We synthesized the modified inhibitor (PD168393sl, Figure 2D) as described in [32]. To perform the labelling reaction, we incubated purified EGFR with an equimolar amount of PD168393sl, as previously done for the ATPsl sample. Figure 2B (red) shows that the spin labelled inhibitor fits nicely into the ATP binding cleft of EGFR. For this compound, high labelling degrees of >90 % were typically determined with room temperature X-band cw-EPR spectroscopy [32]. Figure 3B shows a PELDOR time trace of a 1:1 EGFR-PD168393 complex that was specifically recorded as a direct comparison to the ATPsl sample. It is of very good quality with a modulation depth of ~30 % and a clear oscillation that corresponds nicely to the expected distance of the asymmetric EGFR dimer (Fig 3B). This shows that inhibitor-directed spin labelling (IDSL) is a viable option to study suitable proteins with PELDOR in a highly precise but at the same time minimally invasive way.

Obviously, the IDSL approach limits the placement of the spin label to one position, the active site in the case of EGFR. So, what can be learned from a single PELDOR distance? As mentioned above, we aimed to study the structure of the asymmetric EGFR dimer in solution. Fortunately, a large amount of biochemical data was already available, such as mutants that were known to destabilize the dimerization interface of the crystallographically observed, asymmetric EGFR dimer [28,33].

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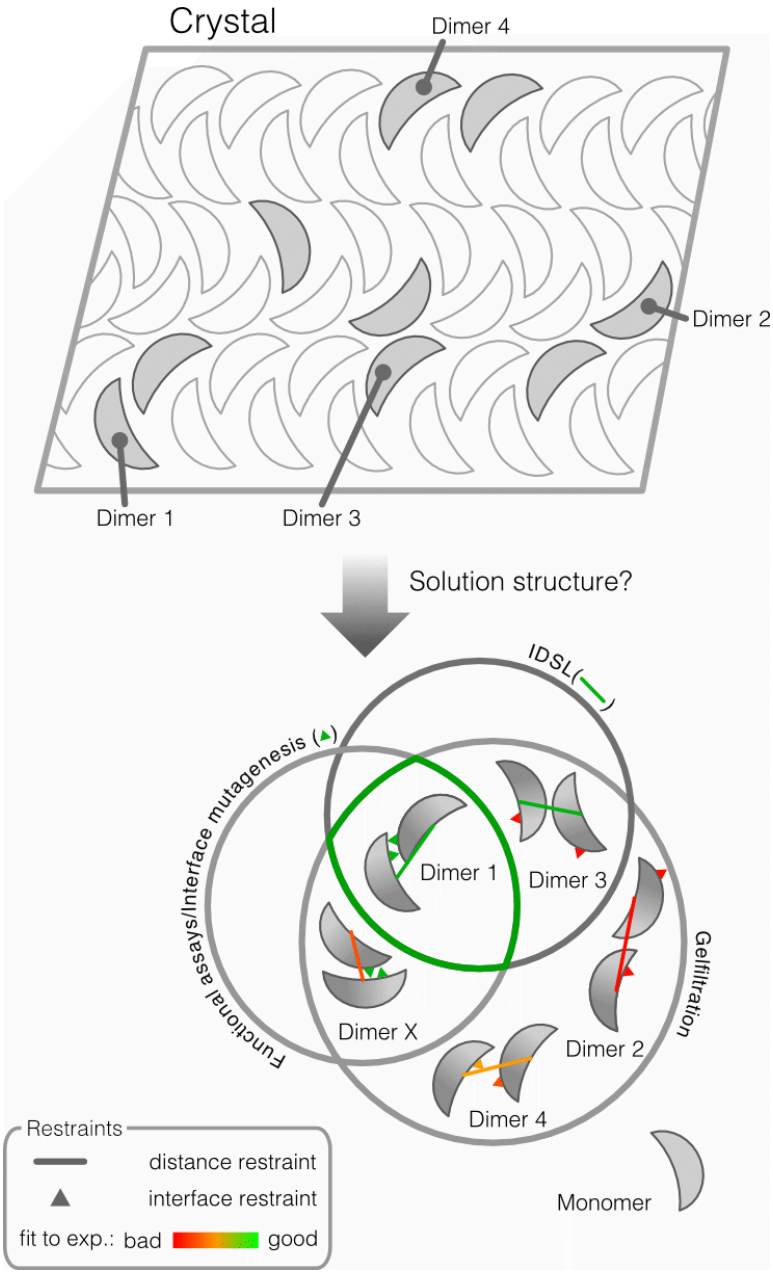

Figure 4: IDSL as a tool in integrative structural biology: What is the solution structure of EGFR? From crystal packing analysis, various oligomeric states are possible (Monomer and dimers 1-4). Also, it is always possible that an additional dimer is present ("Dimer X"), which exists in solution but is not incorporated into the crystal lattice. Note that a hypothetical crystal is shown for visualization purposes. In this example, gel filtration experiments rule out the monomeric structure but cannot distinguish between the different possible dimers. Interface mutations indicate whether certain amino acids are within the dimer interface. They do not give information about the overall structure of the protein (compare Dimer 1 and Dimer X). IDSL provides a single long-distance restraint, which together with the other techniques narrows down the possible dimer structures.

By combining such prior knowledge with PELDOR distance measurements and gel filtration analyses, it was possible to deduce that the distance measured by PELDOR indeed corresponds to the proposed active state dimer, therefore strongly indicating that the crystallographically observed dimer also exists in solution [32] (Figure 4). Thus, while the information provided by IDSL (i.e. a single PELDOR distance) is limited on its own, it is very powerful in combination with orthologous techniques such as gel filtration or mutagenesis studies (Figure 4). It should be noted that both IDSL and spin labelled natural substrates are not suited to study intramolecular conformational changes of monomeric proteins, because two or more spin centers are needed to apply PELDOR spectroscopy, while typically only one inhibitor/substrate binding site exists in a monomeric protein. But, IDSL can be used to study complexes of these molecules. The spin labelled inhibitor can also be used in combination with conventional spin labels, for example to place a label close to the active site without introducing mutations into this usually very delicate environment. What are further potential targets that can be studied with the IDSL approach? The plethora of receptor tyrosine kinases for instance are obviously well suited. All of them form dimers in the activated state. Due to their therapeutic relevance, highly specific inhibitors are available for most of them. Noteworthy, the latest generations of inhibitors are often covalent inhibitors, just as PD168393. Another class of proteins, which could potentially be studied by this approach are cytosolic kinases. On one hand, some of them form homodimers, e.g. BRAF [45]. On the other hand, they often activate so-called downstream kinases by phosphorylation with which they form a transient heteromeric complex, e.g. BRAF forms a complex with MEK1[46]. Since inhibitors are available for many of these proteins, IDSL should be useful to obtain structural information on such complexes. The IDSL approach is of course not limited to kinases but is applicable to any macromolecule for which suitable inhibitors together with structural information about their mode of binding exists. To name only a few examples from a long and diverse list of possible targets and inhibitors: G protein-coupled receptors (e.g. $P2Y_{12}$ and its covalent inhibitor Clopidogrel (Sanofi)), enzymes (e.g. acetylcholinesterase is covalently inhibited by rivastigmine, Novartis), ion pumps (omeprazole (AstraZeneca) inhibits the H^+/K^+ ATPase) or large complexes such as the proteasome (bortezomib (Millenium)) [47]. Further, in combination with spin counting and quantitative cw-EPR spectroscopy, IDSL might be useful to determine the number of inhibitor-labelled protomers in an oligomeric complex and, thus, to determine its stoichiometry [48]. Of course, for each particular inhibitor, possibilities to chemically attach a spin center without significantly diminishing the binding affinity have to be explored. Any structural information about the

binding mode of the inhibitor would be helpful at this stage. Depending on the accessibility of the inhibitor in the target molecule it would even be possible to prepare differently labelled versions of the inhibitor, allowing to gain more spatial information from the PELDOR measurements.

The ultimate goal of structural biology is to study the function of macromolecules in the context of their native environment, i.e. inside living cells or inside the native cellular membrane. This is by definition not possible by crystallography and is currently also not possible to achieve via NMR spectroscopy. Cryo-electron tomography is a technique that has the potential to achieve this goal but is (currently) not able to provide the necessary sub-nanometer resolution [49]. EPR spectroscopy has already successfully been used to measure distances in frozen cells [10,50]. Thus, just as it is routinely done *in vitro*, it can in principle be used to validate a particular structural model in the context of the living cell. However, the approach is still plagued by the fact that the workhorse spin label MTSSL is not well suited for this kind of measurements, because of its instability inside the mostly reducing conditions of the cytosol and the general difficulty in selectively attaching the spin label *in vivo*. The unnatural amino acid approach has been used to solve this problem, but as mentioned above is not suited for all targets [27,51]. The IDSL technique is potentially very useful for such studies. Assuming the spin labelled high-affinity inhibitor can be taken up by the cell, the probe will home-in on its target and the distance measurement can be performed. Note that off-target reactions are possible and have to be considered during data interpretation [52]. Also, with IDSL, it is of course important to use spin labels that are resistant towards the reducing conditions of the cellular environment. While this is the case for trityl- or gadolinium spin labels, they are likely too large to be applied in this way. A possible alternative are ethyl variants of MTSSL, which have been shown to be very stable in cells and are only slightly larger than MTSSL [53]. In summary, the concept of IDSL appears as a very powerful tool to provide otherwise difficult to acquire long-distance restraints for integrative structural biology approaches.

Keywords: PELDOR • spin labelling • EGFR• IDSL

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