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main of the opposing monomer. Since the C-terminal α-helix is connected to a loop that directly contacts the nicotinamide ring, the helix could serve as the lever that allows the DNA binding domains to reorient in response to NADH/NAD$^+$ exchange.

While the structure of NADH bound T-Rex adds new insight into NADH/NAD$^+$ regulation of gene expression, it raises new questions as well. What type of conformational change occurs upon NADH/NAD$^+$ exchange, what is the role of the C-terminal α-helix, and what are the NADH and NAD$^+$ dissociation constants? It has been reported that CtBP, which functions as both a dehydrogenase and a eukaryotic transcriptional corepressor, as well as the NPAS/BMAL transcription factors also are regulated by dinucleotide ratios (Rutter et al., 2001; Zhang et al., 2002). How many other proteins are modulated by fluctuations in NADH/NAD$^+$ ratios, and what other conformational changes are brought about by dinucleotide binding? Did even Tyrannosaurus Rex contain a T-Rex protein? Finally, two critical questions in all organisms with putative NADH/NAD$^+$ sensors are: how do the concentrations of free NADH and NAD$^+$ compare to the binding affinities of the sensor proteins and how do NADH/NAD$^+$ ratios change in different oxygen environments, cells types, and subcellular compartments? It is likely that Rex will serve as a paradigm for answering many of these questions.

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Resolving Protein Structure Dynamically

In this issue of *Structure*, Rajagopal et al. (2005) report further innovations in the X-ray diffraction analysis of the dynamical changes in protein conformation; they use these methods to resolve the light-activated changes in the conformation of Photoactive Yellow Protein. This approach allows a straightforward reinforcement of X-ray diffraction data with spectroscopic data.

The paper of Rajagopal et al. in this issue (2005) is the most recent one in a series of seminal papers (Anderson et al., 2004; Genick et al., 1997; Perman et al., 1998; Rajagopal et al., 2004a; Ren et al., 2001; Schmidt et al., 2003, 2004) from the group of Keith Moffat (at the University of Chicago) in which they describe the resolution of the sequence of functional light-induced changes in the conformation of the photoreceptor protein Photoactive Yellow Protein (PYP). These studies were made possible through the availability of ultrashort (picosecond) X-ray pulses from a synchrotron beam line. The use of an advanced X-ray shutter allowed selection of a suitable repetition rate (Moffat et al., 1989). The time-resolved studies were initiated with an analysis of the relaxation of the structure of a cw-light accumulated millisecond intermediate of PYP (Genick et al., 1997); the use of laser excitation subsequently allowed nanosecond time-resolution (Anderson et al., 2004; Perman et al., 1998; Rajagopal et al., 2004a; Ren et al., 2001; Schmidt et al., 2003, 2004).

To evaluate the very large data set obtained, it required extensive and innovative data averaging (compare Perman et al., 1998 and Ren et al., 2001) and data reduction (compare Perman et al., 1998 and Schmidt et al., 2003). Unlike spectroscopic techniques (such as optical or infrared spectroscopy), however, time-resolved X-ray diffraction allows direct visualization of the dynamics of the entire chemical structure. Moreover, a crucial additional innovation was achieved by the Moffat group through application of a data analysis method based on singular value decomposition (SVD), a method that has already proven very useful in the analyses of data from optical spectroscopy (*e.g.*, van Stokkum et al., 2004); note that the SVD analysis has to be applied to data in real space (Rajagopal et al., 2005; Schmidt et al., 2003, 2004). This data analysis method provides a way to extract the structure of the intermediates involved and the respective time con-

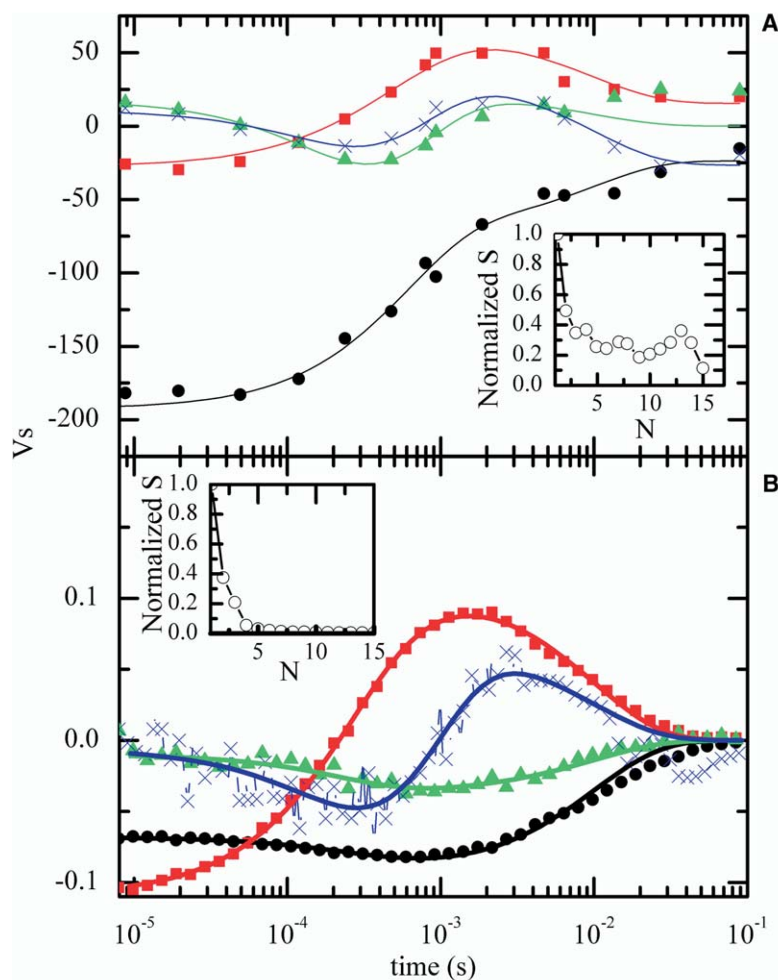

Figure 1. Time Dependence of the First Four (Significant) Right Singular Vectors Derived from the Experimental Data of Time-Resolved X-Ray Diffraction (Symbols in Plot (A) – Adapted from 1) and UV-Visible Transient Absorption Spectroscopy (Symbols in Plot B)

The circles, squares, triangles, and crosses depict first, second, third, and fourth right singular vector, respectively. Corresponding singular values are shown in the insets of Figures 1A and 1B. The lines represent results of a global fit by a sum of three exponential functions with the following time constants for the X-ray data set (and the values for the UV-Vis data between brackets): 0.2 (0.25) ms, 0.62 (0.71) ms, and 10.13 (10.21) ms.

stants of the protein under study, *i.e.*, to formulate a chemical kinetic mechanism. Application of "target analysis" (van Stokkum et al., 2004) may even further optimize data analysis in this type of experiment. The impact of this study goes far beyond improving our understanding of PYP only: it provides, for instance, a very challenging data set with which to compare future computational predictions for a complete protein catalytic cycle.

Presumably because of its faster photocycle, and the more limited structural changes elicited, so far the most detailed analyses have been reported on the E46Q variant of PYP. Similar studies on the wild-type protein are in progress. Earlier, already a simplified SVD-based analysis of this data set was published (Rajagopal et al., 2004b), and the same data set was subsequently used to derive a gross structure of the *p*-coumaryl chromophore of PYP in an "early/red-shifted" and a "late/blue-shifted" intermediate (Anderson et al., 2004). The current analysis, however, fully exploits the strength of the SVD procedure to derive a chemically interpretable mechanism of progression of PYP through its photocycle. The data do reveal a limited set of discrete intermediates (Schmidt et al., 2003), of which the spatial structure has been solved at high resolution. Their mutual connections, however, were not unequivocally resolved: a number of different reaction mechanisms remain, each compatible with the data set recorded.

In the current study, impressive progress is reported in our insight in the light-induced conformational transitions in PYP, from nanoseconds up to complete recovery (which is complete within one second in E46Q-PYP). Besides the configurational transitions in the chromophore (*i.e. trans* → *cis* → *trans*), specific side chain movements, and restructuring of the hydrogen-bonding network around the chromophore, it is revealed that small conformational changes radiate like a protein quake from the chromophore binding pocket to the exterior of the protein. One should keep in mind though, that this walk of PYP through its free energy landscape of protein conformation is significantly affected by the constraints of the crystal lattice (compare Derix et al., 2003 and Rubinstenn et al., 1998). Similar constraints may, or may not, be present in vivo.

So far there are two practical parameters that restrict the accuracy of conclusions that can be derived from these time-resolved diffraction experiments. These are the relatively scarce time sampling (up to now only several dozen time points have been analyzed in the time-

scale ranging from nanoseconds to seconds) and the relatively poor signal-to-noise ratio. This has led to a remaining ambiguity in the interpretation of the experimental data. As pointed out above, the analysis of the experimental data obtained with the E46Q mutant of PYP (as well as the previous study of the wild-type PYP; Schmidt et al., 2004) did not allow the selection of a fully definite kinetic model for its photocycle.

Significantly, further progress on both aspects may be achieved by combining the method of time-resolved X-ray diffraction with time-resolved UV/Vis spectroscopy on single crystals. The latter method provides both a substantially better temporal resolution and sampling, and a better signal-to-noise level. Together with the straightforward application of the "target analysis" method on the spectroscopic data, this may allow one to select an unambiguous chemical kinetic scheme for the photocycle of E46Q-PYP. Furthermore, transient UV/Vis spectroscopy can also bridge observations made on PYP in different molecular environments (compare Genick et al., 1997; Rajagopal et al., 2005; Rubinstenn, et al., 1998; Xie et al., 2001).

An example of comparison of spectroscopic and time-resolved X-ray diffraction data is given in Figure 1, where the time dependence of the first four (*i.e.*, the significant ones) right singular vectors, obtained from a complete diffraction data set (Figure 1A, adapted from Rajagopal et al., 2005), and from a UV/Vis transient absorption measurement on a single crystal of PYP (Figure 1B), is shown. The corresponding singular vectors are strikingly similar. Furthermore, the global fit of the right singular vectors by a sum of three exponentials yields almost identical time constants for the two data sets, as explained in the figure caption. The relative signal-to-noise level in these two data sets (see above) can be directly compared by comparison of the magnitude of the singular values at the high value numbers (see insets of the Figure 1A and 1B). Significantly, future detailed "target analyses," of combined diffraction- and UV/Vis spectroscopic data, may well lead to the formulation of a ***unique*** kinetic mechanism for progression through the PYP photocycle.

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