Biol. Chem., Vol. 380, pp. 931 – 935, July/August 1999

Minireview

Molecular Reaction Mechanisms of Proteins Monitored by Time-Resolved FTIR-Spectroscopy

Klaus Gerwert

Ruhr-Universität Bochum, Lehrstuhl für Biophysik,
D-44780 Bochum, Germany

Time-resolved FTIR difference spectroscopy can provide a valuable insight into the molecular reaction mechanisms of proteins, especially membrane proteins. Isotopic labeling and site-directed mutagenesis allows an unequivocal assignment of IR absorption bands. Studies are presented which give insight into the proton pump mechanisms of proteins, especially bacteriorhodopsin. H-bonded network proton transfer via internal water molecules seems to be a general feature in proteins, also found in cytochrome *c* oxidase. Using caged GTP the intrinsic and GAP catalyzed GTPase activity of H-ras p21 is studied. Furthermore, protein folding reactions can be recorded with ns time-resolution.

Key words: Bacteriorhodopsin / Caged GTP / FTIR / H-ras p21 / Proton transfer.

Introduction

Time-resolved FT-IR difference spectroscopy has been established as a new biophysical tool for the investigation of molecular reaction mechanisms of proteins at the atomic level (Gerwert *et al*., 1992).

The infrared spectrum of a protein is dominated by its peptide backbone amide I (C=O) and amide II (C–N, NH) vibrations in Figure 1 at 1660 cm^{-1} and 1550 cm^{-1}. In addition to the strong amide I and amide II bands water also contributes largely to the background absorption (1650 cm^{-1}) (Colthup *et al*., 1990). The major problem in measuring reactions consists in selecting the small absorption bands of the molecular groups which undergo reactions from the large background absorption of water and of the quiescent entire protein. The absorbance bands are selected by the performance of difference spectra between spectra of the protein in its ground state and in an activated state. Such measurements require highly sensitive instrumentation.

A further important step is the assignment of the bands to individual groups of the protein. This is performed by using isotopic labeling or by using site-directed mutated proteins. Also individual residues and the peptide chain can be site-detected isotopically labelled via genetic techniques (Ludlam *et al*., 1995). This combination of FTIR with molecular biology methods allows an unequivocal assignment.

In protein chemistry the macromolecule studied most intensely thus far by time-resolved FT-IR difference spectroscopy is the light-driven proton pump bacteriorhodopsin. Many research groups worldwide have contributed to the understanding of the mechanism of this membrane protein (Grigorieff *et al*., 1996). For reviews on FT-IR studies on bacteriorhodopsin, see Braiman *et al*. (1988); Gerwert (1992) and Siebert (1993). Time-resolved FT-IR spectroscopy has furthermore provided a detailed picture of the light-induced electron transfer mechanism of bacterial photosynthetic reaction centers. For reviews and recent FT-IR work see Robert *et al*. (1989), Mäntele (1993) and Brudler *et al*. (1994). Besides photobiological proteins, time-resolved FT-IR spectroscopy can also be

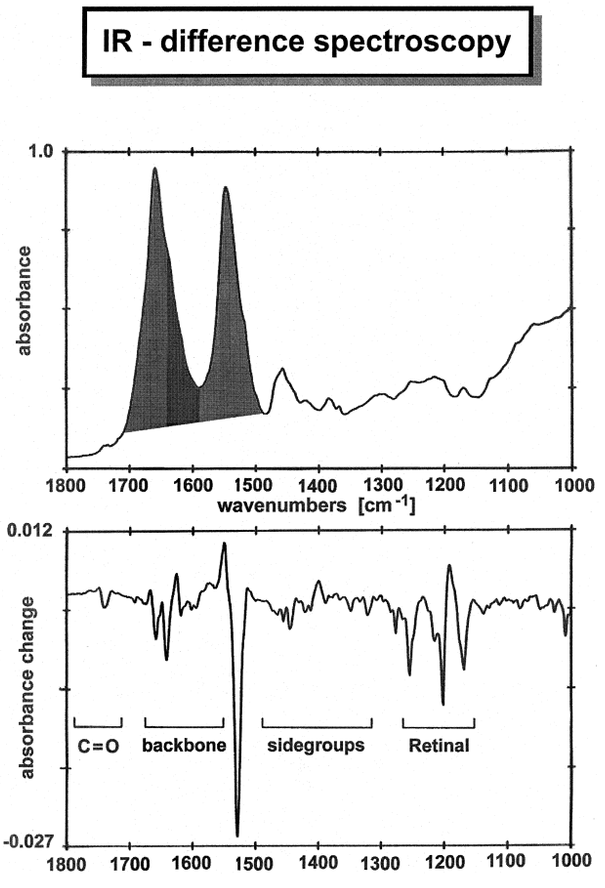

Fig. 1 An IR Absorbance Spectrum of a Hydrated Protein (Bacteriorhodopsin) and a Typical Difference Spectrum.

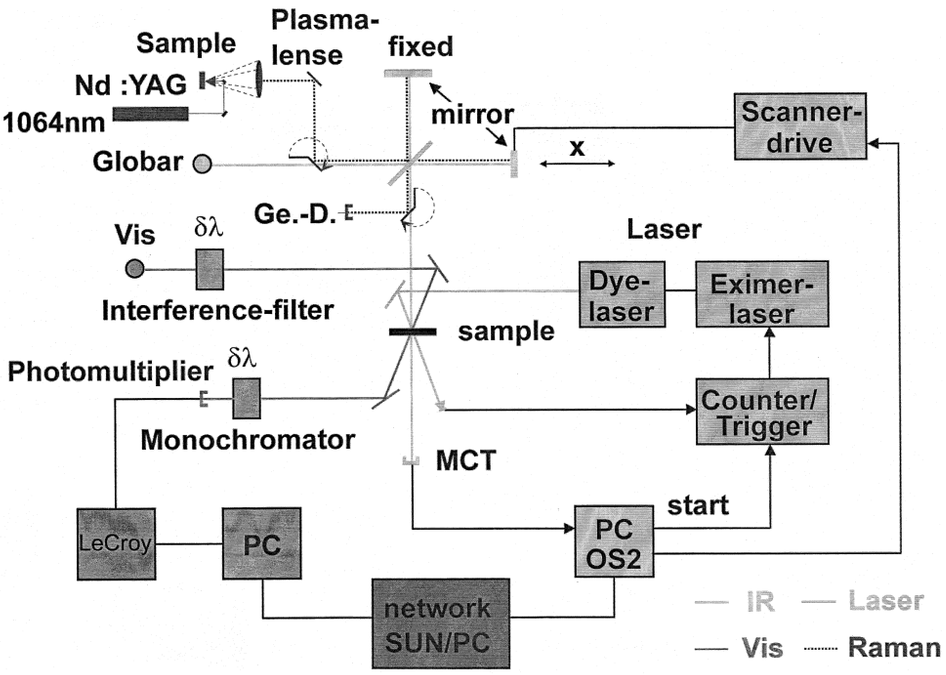

Fig. 2 Schematic Representation of the FTIR Experimental Setup.
The FT-IR apparatus is equipped with globar, beamsplitter, mirrors, controller, detector and preamplifier connected to a 200 kHz and a 200 MHz transient recorder; a photolysis setup with light source, interference filters, monochromator, photomultiplier and transient recorder; and an excimer pumped dye laser system to activate the sample. The VIS and IR data are transferred to a workstation (SUN)/PC network for kinetic analysis.

applied to proteins without an intrinsic chromophore by using photolabile trigger compounds (McGray *et al*., 1989; Cepus *et al*., 1998). Using 'caged' GTP the molecular GTPase mechanism of the oncogenic protein H-ras p21, a molecule that plays a central role in the growth of cancer cells (Wittinghofer and Pai, 1991), can be monitored (Cepus *et al*., 1998). Another example is 'caged' Ca^{2+}, by which the molecular mechanism of the Ca^{2+}-ATPase is investigated (Barth *et al*., 1991; Troullier *et al*., 1996). Furthermore 'caged' electrons are used to study redox reactions in cytochrome oxidases (Lübben *et al*., 1996, 1999).

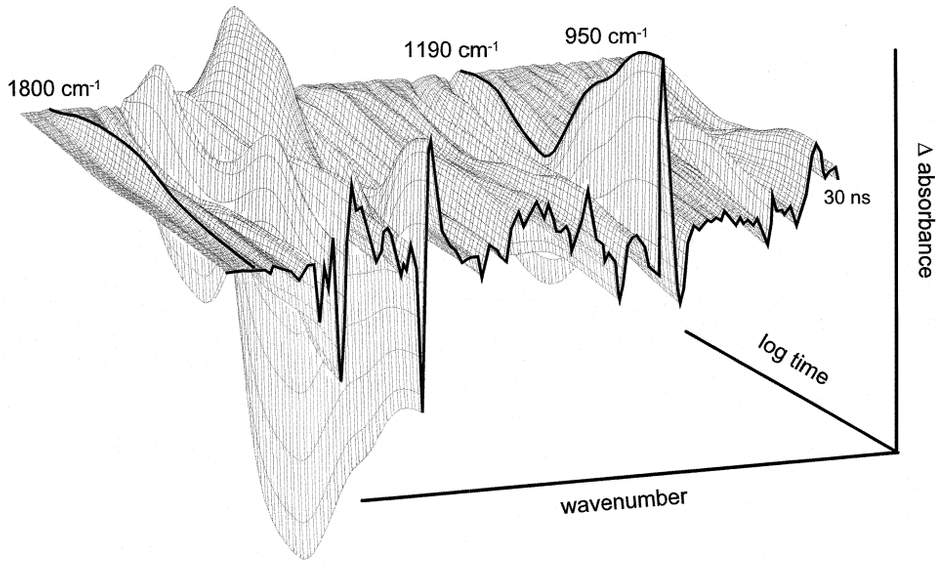

Fig. 3 IR Absorbance Changes during One Photocyle of Bacteriorhodopsin.
A three-dimensional representation of the IR absorbance changes between 1800 cm^{-1} and 1000 cm^{-1} with 30 ns time-resolution and 3 cm^{-1} spectral resolution accompanying bacteriorhodopsin's photocycle as revealed by a global-fit analysis. The time axis has a logarithmic scale in order to show the complete bacteriorhodopsin photocycle from 30 ns up to 10 ms in one representation.

A typical experimental setup is shown in Figure 2. This setup allows time-resolved FTIR, VIS absorbance change and FT Raman measurements. Various time-resolved FT-IR techniques have been developed: the rapid-scan technique, which gives millisecond time resolution (Braiman *et al*., 1987; Gerwert, 1988), the stroboscope technique, which gives microsecond time resolution (Gerwert *et al*., 1990; Braiman *et al*., 1991; Souvignier *et al.*, 1992) and the step-scan technique, which gives nanosecond time resolution (Uhmann *et al*., 1991; Palmer *et al*., 1991; Rammelsberg *et al.*, 1997). Here bacteriorhodopsin is presented as an example.

Bacteriorhodopsin

After light-excitation of bacteriorhodopsin's light-adapted ground-state BR_{570} a photocycle starts with the intermediates J_{610}, K_{590}, L_{550}, M_{410}, N_{530} and O_{640} distinguished by the different absorption maxima given by the indices. During the rise of the M-intermediate a proton is released to the extracellular side, and during the M-decay a proton is taken up from the cytoplasmic side. This creates a chemic-osmotic proton gradient across the membrane.

The Thermal Reactions: K → L → M → N → O → BR

The absorbance changes accompanying this reaction pathway can be monitored under physiological conditions by the step scan FTIR technique with a time resolution of 30 ns (Rammelsberg *et al*., 1997).

In Figure 3 the IR absorbance changes between 1800 cm^{-1} and 1000 cm^{-1} during the photocycle are shown in a three-dimensional representation. Beyond a background absorbance of up to one absorbance unit, changes on the order of 10^{-3} to 10^{-4} are monitored with 3 cm^{-1} spectral resolution and 30 ns time resolution.

The appearance of the C-C stretching vibration band at 1190 cm^{-1} indicates the all-*trans* to 13-*cis* isomerization of retinal. It takes place within 450 fs and is not time resolved here. Its disappearance at about 200 μs indicates the deprotonation of the Schiff base. This loss of charge of the Schiff base greatly reduces the IR-absorbance of the chromophore. [The Schiff base connects the retinal to Lys216 of the protein (Figure 4).] The deprotonation kinetics of the Schiff base agree nicely with the protonation kinetics of the counterion Asp85, which can be followed at 1761 cm^{-1} (Gerwert *et al*., 1989) (Figure 4). Recently, for the proton release pathway to the protein surface a protonated hydrogen bonded network has been identified (Figure 4) (Rammelsberg *et al*., 1998). The reappearance of the band at 1190 cm^{-1} in the millisecond time domain indicates the reprotonation of the Schiff base. It is reprotonated by Asp96 (Gerwert *et al*., 1989; le Coutre *et al*., 1995). The final disappearance at 1190 cm^{-1} shows the chromophore's relaxation to the all-trans BR-ground state configuration.

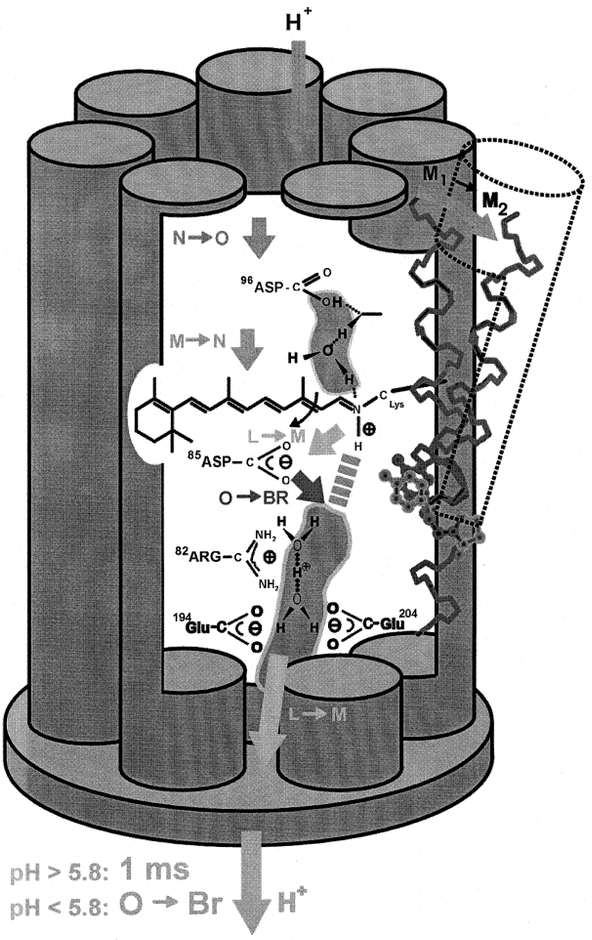

Fig. 4 The Current Model of the Proton Pump Mechanism of Bacteriorhodopsin.
After the light-induced all-*trans* to 13-*cis* retinal isomerization in the BR to K transition, the Schiff base proton is transferred to Asp85 in the L to M transition. Simultaneously a proton is released from a protonated H-bonded network spanned by internal water molecules to the extracellular site. This network is controlled by Arg 82, Glu204 and Glu194. Asp85 reprotonates the network in the O to BR reaction. The Schiff base is oriented in the M_1 to M_2 transition from the proton release site to the proton uptake site by a small backbone movement of helix F and determines thereby the vectoriality of proton pump. A larger backbone movement is observed in the M to N transition as compared to the M_1 to M_2 transition. Asp96 reprotonates the Schiff base in the M to N transition. Asp96 itself is reprotonated from the cytoplasmic site in the N to O transition. (For references see text).

Based on the FTIR experiments a detailed model of the lightdriven proton pump bacteriorhodopsin has been elucidated and is presented in Figure 4.

H-ras p21

The small GTP-binding protein H-ras p21 plays a central role in signaling leading to cell-growth (Wittinghofer and Pai, 1991). P21 appears to act as a switch: in the GTP bound form a signal is given to an effector molecule which is part of a cascade leading to cell proliferation and differ-

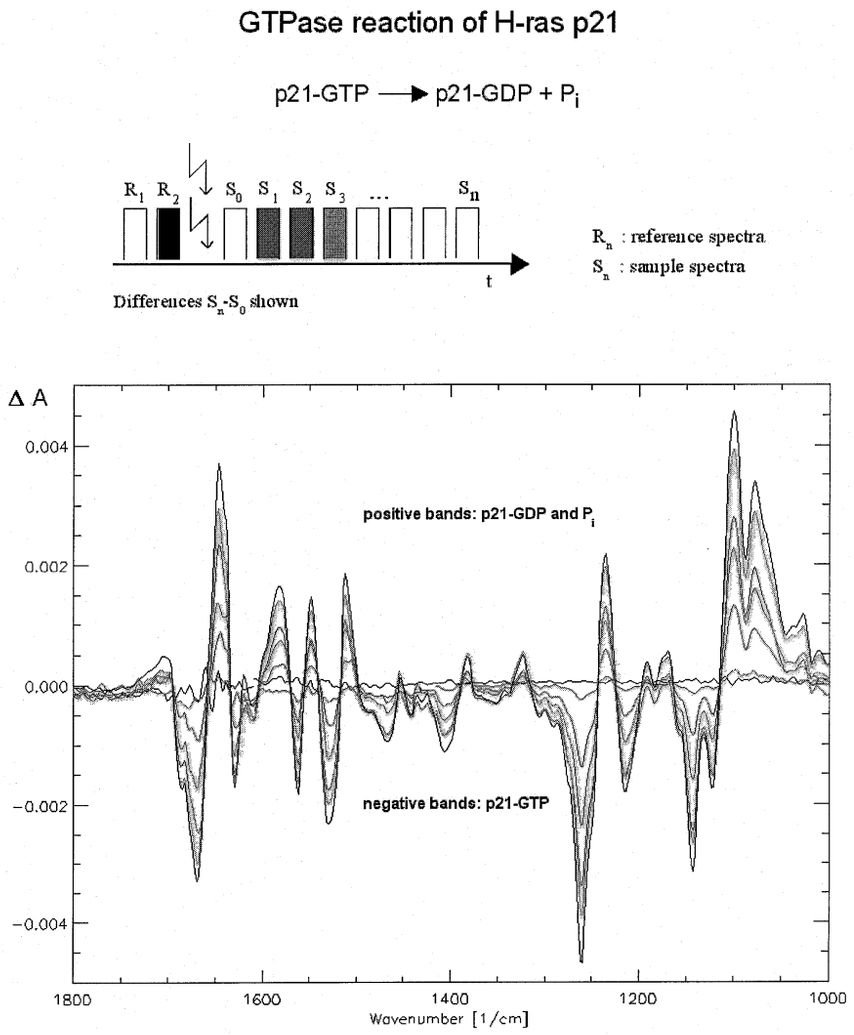

Fig. 5 Principle of the Measurement of the Intrinsic p21 GTPase Reaction.
GTP is released from caged GTP using several UV flashes and GTPase activity starts. Difference spectra are taken between the first spectrum recorded directly after GTP (S_0) is released and spectra taken during the following GTPase reaction (S_i) (upper part of the Figure). The IR absorbance changes are represented in the lower part of the Figure.

entiation. The intrinsic GTPase activity is accelerated by GAP (GTPase activating protein). The transition from the active GTP bound to the inactive GDP bound form is accompanied by a conformational change. In order to specify in more detail the structural changes occurring in the GTP to GDP reaction, we have started complementary to the X-ray studies investigations by time-resolved FTIR difference spectroscopy.

The reaction is studied using caged GTP complexed to p21 instead of GTP to prevent hydrolysis. Using an intensive UV flash (308 nm, 100 mJ) the caged group is photolysed, GTP released and GTPase activity is precisely started. In Figure 5 difference spectra between p21-GTP and finally p21-GDP are shown. The region between 1300 and 900 cm^{-1} is dominated by phosphate bands. Using isotopically labeled caged GTP the phosphate vibrations are asigned, for example the band at 1144 cm^{-1} is assigned to the γ-Phosphate vibration (Cepus *et al.*, 1998). Its absorbance change can be used as marker hand for the GTPase kinetics.

The results on p21 show that molecular reaction mechanisms of proteins without intrinsic chromophore can now be investigated with time-resolved FTIR. In future studies, in addition to the intrinsic mechanism of p21 the GAP catalyzed GTPase mechanism and the interaction with the downstream effector Raf will be examined.

The presented results demonstrate that time resolved FTIR difference spectroscopy in combination with molecular biological methods allows a detailed analysis of the molecular reaction mechanism of proteins. The use of caged compounds provides a powerful tool also for non-photobiological proteins.

Acknowledgement

I would like to thank my coworkers Dr. Benedikt Heßling, Dr. Ronald Brudler, Dr. Valentin Cepus, and Robin Rammelsberg for contributions to this work.
The financial support of the DFG in the SFB394, Teilprojekt B1 and C2,and SFB 480, TP C3, is gratefully acknowledged.

References

Barth, A., Mäntele, W., and Kreutz, W. (1991). Infrared spectroscopic signals arising from ligand binding and conformational changes in the catalytic cycle of sarcoplasmic reticulum calcium ATPase. Biochim. Biophys. Acta *1057*, 115.

Braiman, M.S., and Rothschild, K. (1988). Fourier transform infrared techniques for probing membrane protein structure. Ann. Rev. Biophys. Chem. *17*, 541.

Braiman, M.S., Ahl, P.L., and Rothschild, K.J. (1987). Millisecond Fourier-transform infrared difference spectra of bacteriorhodopsin's M412 photoproduct. Proc. Natl. Acad. Sci. USA *84*, 5221.

Braiman, M.S., Bousche, O., and Rothschild, K.J. (1991). Protein dynamics in the bacteriorhodopsin photocycle: submillisecond Fourier transform infrared spectra of the L, M, and N photointermediates. Proc. Natl. Acad. Sci. USA *88*, 2388.

Brudler, R., de Groot, H.J.M., van Liemt, W.B.S., Steggerda, W.F., Esmeijer, R., Gast, P., Hoff, A.J., Lugtenburg, J., and Gerwert, K. (1994). Asymmetric binding of the 1- and 4-C=O groups of Q_A in Rhodobacter sphaeroides R26 reaction centres monitored by Fourier transform infra-red spectroscopy using site-specific isotopically labelled ubiquinone-10. EMBO J. *13*, 5523.

Cepus, V., Ulbrich, C., Allin, Chr., Troullier, A., and Gerwert, K. (1998). FTIR photolysis studies of caged compounds. Methods in Enzymology *291*, 223.

Cepus, V., Goody, R.S., Scheidig, A.J., and Gerwert, K. (1998). Time-resolved FTIR studies of the GTPase reaction of H-ras p21 reveal a key role for the β-phosphate. Biochemistry *37*, 10263.

Colthup, N.B., Daly L.H., and Wiberley, S.E. (1990). Introduction to Infrared and Raman Spectroscopy, 3rd ed. (Boston, USA: Academic Press).

Gerwert, K. (1988). Intramolekulare Proteindynamik untersucht mit zeitaufgelöster Fourier Transform Infrarot-Differenzspektroskopie. Ber. Bunsenges. Phys. Chem. *92*, 978.

Gerwert, K. (1992). Molecular reaction mechanism of photosynthetic proteins as determined by FTIR-spectroscopy. Biochimica et Biophysica Acta *1101*, 147.

Gerwert, K. (1993). Molecular reaction mechanism of proteins as monitored by time-resolved FTIR spectroscopy. Current Opinion Structural Biology *3*, 769.

Gerwert, K., Hess, B., Soppa, J., and Oesterhelt, D. (1989). The role of Asp 96 in the proton pump mechanism of bacteriorhodopsin. Proc. Natl. Acad. Sci. USA *86*, 4943.

Gerwert, K., Souvignier, G., and Hess, B. (1990). Simultaneous monitoring of light-induced changes in protein side-group protonation, chromophore, isomerization, and backbone motion of bacteriorhodopsin by time-resolved Fourier-transform infrared spectroscopy. Proc. Natl. Acad. Sci. USA, *87*, 9774.

Grigorieff, N., Ceska, T.A., Downing, K.H., Baldwin, J.M., and Henderson, R. (1996). Electron-crystallographic refinement of the structure of bacteriorhodopsin. J. Mol. Biol. *259*, 393.

le Coutre, J., Tittor, J., Oesterhelt, D., and Gerwert, K. (1995). Experimental evidence for hydrogen-bonded network proton transfer in bacteriorhodopsin shown by FTIR spectroscopy using azide as catalyst. Proc. Natl. Acad. Sci. USA *92*, 4962.

Lübben, M., and Gerwert, K. (1996). Redox-FTIR difference spectroscopy using caged electrons reveals contribution of carboxyl groups to the catalytic mechanism of haemcopper oxidases. FEBS Letters *397*, 303.

Lübben, M., Prutsch, A., Mamat, B., and Gerwert, K. (1999). Electron transfer induces side chain conformational changes of Glu-286 from cytochrome bo_3. Biochemistry *38*, 2048.

Ludlam, C.F.C., Sonar, S., Lee, C.-P., Coleman, M., Herzfeld, J., RajBhandary, U.L., and Rothschild,K. (1995). Site-directed isotope labeling and ATR-FTIR difference spectroscopy of bacteriorhodopsin: the peptide carbonyl group of Tyr185 is structurally active during the bR → N transition. Biochemistry *34*, 2–6.

McGray, J.A., and Trentham, D. (1989). Properties and uses of photoreactive caged compounds. Annu. Rev. Biophys. Biophys. Chem. *18*, 239.

Mäntele, W. (1993). Infrared vibrational spectroscopy of the photosynthetic reaction. In: The Photosynthetic Reaction Center Vol. II J. Deisenhofer and J. Norris, eds. (New York: Academic Press), p. 239.

Palmer, R.A, Manning, C.J., Chao, J.L., Noda, I., Dorwrey, A.E., and Marcott, C. (1991). Application of step-scan interferometry to two-dimensional Fourier transform infrared (2D-FT-IR) correlation spectroscopy. Appl. Spectrosc. *45*, 12.

Rammelsberg, R., Heßling, B., Chorongiewski, H., and Gerwert, K. (1997). Molecular reaction mechanisms of proteins monitored by nanosecond step-scan FT-IR difference spectroscopy. Applied Spectroscopy *51*, 558.

Rammelsberg, R., Huhn, G. Lübben, M., and Gerwert, K. (1998). Bacteriorhodopsin's intramolecular proton-release pathway consists of a hydrogen-bonded network. Biochemistry *37*, 5001.

Robert, B., Nabedryk, E., and Lutz, M. (1989). In: Time Resolved Spectroscopy, R.J.H. Clark and R.E. Hester, eds. (New York: Wiley)

Siebert, F. (1993). In: Biomolecular Spectroscopy, Part A, R.J.H. Clark and R.E. Hester, eds. (Chichester, UK: Wiley).

Souvignier, G., and Gerwert, K. (1992). The proton uptake reactions of Bacteriorhodopsin determined by time-resolved stroboscopic-FTIR-spectroscopy. Biophy. J. *63*, 1393.

Troullier, A., Gerwert, K., and Dupont, Y. (1996). A time resolved FTIR difference spectroscopy study of the SR Ca^{2+}-ATPase: kinetics of the high affinity calcium binding at low temperature. Biophys. J. *71*, 2970.

Uhmann, W., Becker, A., Taran, C., and Siebert, F. (1991). Time-resolved FT-IR absorption spectroscopy using a step-scan interferometer. Appl. Spectrosc. *45*, 390.

Wittinghofer, A., and Pai, E.F. (1991). The structure of Ras protein: A model for a universal molecular switch. Trends Biochem. Sci. *16*, 382.