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Fourier transform infrared spectroscopic characterization of a photolabile precursor of glutamate

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Abstract Recently, it has been demonstrated that Fourier transform infrared spectroscopy (FTIR) detects conformational changes in the glutamate receptor ligand-binding domain that are associated with agonist binding. Combined with flash photolysis, this observation offers the prospect of following conformational changes at individual protein and agonist moieties in parallel and with high temporal resolution. Here, we demonstrate that γ(α-carboxy-2-nitrobenzyl) glutamate (caged glutamate) does not interact with the protein, and that following photolysis with UV light the FTIR difference spectrum indicated changes in the protein tertiary and secondary interactions. These changes were similar to those observed for the protein upon addition of free glutamate. Thus, caged glutamate and its photolysis by-products are inert in this system, whereas the released glutamate exhibits full activity. Difference spectra of caged glutamate and of reaction analogs permitted identification of and correction for FTIR signals arising from the photolytic reaction and confirmed that its products are indeed glutamate and 2-nitrosophenyl glyoxalic acid. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Fourier transform infrared spectroscopy; Caged glutamate; Glutamate receptor

1. Introduction

Rapid photochemical release of biological substrates from photolabile inert precursors (caged compounds) has emerged as a powerful technique for generating rapid concentration jumps [1–5]. This technique has proven particularly useful for studying the kinetics of biological processes that occur on the sub-millisecond time scale. Such processes are inaccessible to alternative methods relying on diffusional mixing, which are characterized by millisecond time delays. The most commonly used photoremovable caging group is the 2-nitrobenzyl group, which is used to cage moieties such as phosphates, amines, carbamates, carbonates, carboxylates, phenols and alcohols [1–3]. Photolysis with UV light cleaves the 2-nitrobenzyl group liberating the caged moiety (Fig. 1) [1,6–8]. The 2-nitrobenzyl caging group has been particularly useful in neurobiology, as many of the compounds active in the central nervous system are small carboxylate-containing compounds, such as the amino acid neurotransmitters. One such caged neurotransmitter is γ(α-carboxy-2-nitrobenzyl) glutamate, which liberates glutamate when photolysed with UV light with a time constant of 12 μs (Fig. 1) [9].

Glutamate acts as an excitatory neurotransmitter in the central nervous system, and upon binding to the ionotropic glutamate receptors (GluR) mediates excitatory signals through the formation of cation-permeable transmembrane ion channels in the receptors [10–13]. At the level of molecular function of the glutamate receptors, caged glutamate has permitted the analysis of the activation and desensitization of GluR with unprecedented temporal resolution; it has also been used to map circuits in the mammalian visual cortex [1,9,14,15]. Using electrophysiological measurements it has been shown that the caged glutamate is biologically inert, i.e. that it induces neither activation nor desensitization, and that the photolytically released glutamate is fully active [9]. However, there has not yet been a direct spectroscopic structural demonstration that the reaction proceeds as proposed for caged glutamate.

Agonist binding to GluR is mediated by an extracellular domain, known as S1S2 [16,17]. This domain has been expressed as a soluble fusion protein for two subunits belonging to the GluR family specific for α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid, namely GluR2 and GluR4 [17–19]. The S1S2 protein retains ligand-binding parameters that are quite similar to those of the intact receptor, thus indicating that these protein segments can be used as models to study the ligand–protein interactions of the receptor [17–21]. Although the kinetics of S1S2 agonist binding have recently been studied by stopped-flow intrinsic fluorescence methods, some binding interactions were too fast to be observed directly [22]. Furthermore, it will be essential to monitor the kinetics of binding interactions and conformational changes at a number of functional groups within the complex in order to obtain a complete picture of the progress of the reaction.

Recently, we have used Fourier transform infrared spectroscopy (FTIR) techniques to identify structural changes associated with glutamate binding to the ligand-binding domain of a glutamate receptor [23]. The use of caged compounds should permit us to follow the kinetics of these changes with higher temporal and spatial resolution. However, it is important to establish that the caged glutamate is 'inert' in this system as well, i.e. that it does not induce structural changes in the protein itself, and that following photolysis, the changes are equivalent to those seen with free glutamate [1]. Furthermore, it is necessary to characterize the IR spectral changes induced by the photolysis reaction in order to distinguish them from signals due to glutamate binding and protein conformational changes. To address these requirements, we have used FTIR, which is extremely sensitive to the strength and nature of

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inter- and intra-molecular interactions, to study the structures of the photolysis products of caged glutamate and to investigate their interactions with the protein.

2. Materials and methods

2.1. Chemicals

Glutamate, nitrobenzene, nitrosobenzene, sodium pyruvate, sodium acetate and sodium acetoxyacetate were purchased from Aldrich (Milwaukee, WI). γ(α-Carboxy-2-nitrobenzyl) glutamate was obtained from Molecular Probes (Eugene, OR).

2.2. Protein preparation and characterization

The GluR4 S1S2 protein was expressed, purified, and characterized as described [24]. In brief, S1S2 was expressed as a secreted construct in the baculovirus system. Following clarification and concentration of the cell culture supernatant, it was purified to homogeneity by immunoaffinity and ion exchange chromatography.

0.25–0.5 mM protein in 25 mM phosphate buffer containing 250 mM NaCl was used for the FTIR measurements. D_2O was used as the solvent to obtain the spectra in the 1450–1800 cm^{-1} region, since water has a large IR absorption band at $\sim$1600 cm^{-1}. However, for studying the S–H stretching vibration, water was used as the solvent.

2.3. FTIR difference spectroscopy

Spectra were recorded with a Nicolet Magna 560 FTIR spectrometer equipped with a TGS detector. The highly stable interferometer permits reliable measurements of very small intensity differences, 10^{-3}–10^{-5} absorbance units. Spectra were collected at 4 cm^{-1} spectral resolution. A modified variable-length sample holder (Aldrich, Milwaukee, WI) with CaF_2 windows was used to obtain the spectra. The modification of the sample holder allowed liquid from a constant-temperature bath to be circulated around the holder, which ensured that the protein solutions were kept at a constant temperature of 15°C. This level of control was required to ensure no shifts or intensity changes in the bands due to temperature fluctuations. A 50-μm path length was used to obtain the spectra in the 1450–1800 cm^{-1} region, and a 75-μm path length was used for studying the S–H vibration. These path lengths ensured that the absorption of the most intense band in the region of interest was less than one absorption unit, thereby ensuring linear operation of the IR detector.

The FTIR spectra were collected in blocks of 100–500 scans, and summed to yield a final spectrum containing 1000–4000 scans. Data manipulation and analysis was carried out using Grams software (Galactic Industries, Salem, NH). The caged glutamate was photolysed in the FTIR sample holder using UV light (355 nm) from a NdYAG laser. The difference spectra were generated by subtracting the spectrum before photolysis from the spectrum after photolysis. Water vapor absorption lines due to incomplete purging of the spectrometer were digitally subtracted from the difference spectrum using a reference water vapor spectrum obtained at the same resolution and collection parameters.

3. Results and discussion

3.1. Photolysis of caged glutamate

The difference FTIR spectrum for caged glutamate in D_2O after and before photolysis is shown in Fig. 2 (trace A). The negative bands in the difference FTIR spectrum correspond to the vibrational modes of the starting material, namely caged α-carboxy-nitrobenzyl glutamate and the positive bands correspond to the vibrational modes due to the photolysis products. Based on the photolysis pathway for the nitrobenzyl derivatives (Fig. 1), the structural changes upon photolysis of caged glutamate would occur at the nitro, carbonyl, and carboxylate moieties (Fig. 1). Hence, the FTIR spectra were obtained in the 1450–1800 cm^{-1} mid-IR region, in which characteristic vibrational modes arising from these chemical moieties are present.

To facilitate the assignments of the difference features and to confirm the photolysis products to be those predicted by the scheme shown in Fig. 1, FTIR spectra were obtained for equimolar quantities of nitrobenzene, acetoxyacetate, nitrosobenzene, pyruvate, and acetate. These serve as model compounds with the same structural changes (Fig. 1) as that observed for the photolysis reaction of the caged glutamate. The sum of the spectra of nitrosobenzene, pyruvate and acetate was used to model the photolysis products, while the sum of the spectra of nitrobenzene and acetoxyacetate was used to model the reactant (caged glutamate). The difference between these two composite spectra (shown in Fig. 2B) has features similar to those in the difference spectrum of the caged glutamate after and before photolysis. This indicates that the chem-

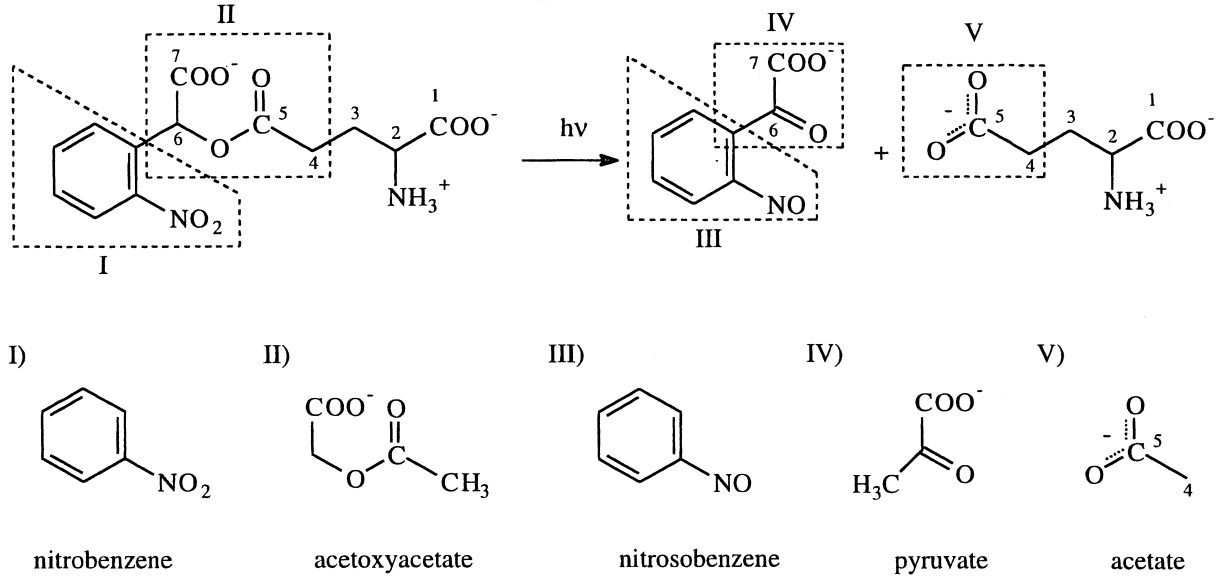

Fig. 1. Photolysis reaction of γ(α-carboxy 2-nitrobenzyl) glutamate which produces glutamate and *o*-nitrosophenyl glyoxalic acid. The model compounds used to study changes in the chemical structures are compounds (I) nitrobenzene, (II) acetoxyacetate, (III) nitrosobenzene, (IV) pyruvate, and (V) acetate.

ical moieties in the model compounds well represent the corresponding moieties in caged glutamate and its photolysis products. This in turn suggests that the photolysis of caged glutamate yields the products predicted by the photolysis reaction pathway shown in Fig. 1.

For specific band assignments of the difference features in the caged glutamate difference spectrum, difference FTIR spectra were obtained between nitrobenzene and nitrosobenzene, and between pyruvate and acetoxyacetate. Based on the frequency and on the difference spectrum between nitrosobenzene and nitrobenzene (Fig. 2C) the negative band at 1531 cm^{-1}, in the difference spectrum for the caged glutamate (Fig. 2A), can be assigned to the asymmetric NO_2 vibration (ν_{asymNO_2}). This assignment is also consistent with FTIR studies of the ^{15}N isotopomer of 1-(2-nitrophenyl) ethyl derivative of ATP (caged ATP) [8]. By comparing the difference spectrum of the caged glutamate to the difference spectrum between pyruvate and acetoxyacetate (Fig. 2D), the positive band at 1635 cm^{-1} can be assigned to the 7C asymmetric carboxylate vibration (ν_{asym7C}) in the *o*-nitrosophenyl glyoxalic acid, and the corresponding mode for the caged glutamate is at 1616 cm^{-1} thus giving rise to the negative band at this frequency. The upshifts of the ν_{asym7C} bands in the difference spectrum of caged glutamate (1639 and 1616 cm^{-1}; Fig. 2A) relative to those in the model compound difference spectrum (1625 and 1600 cm^{-1}, respectively; Fig. 2D), can be attributed

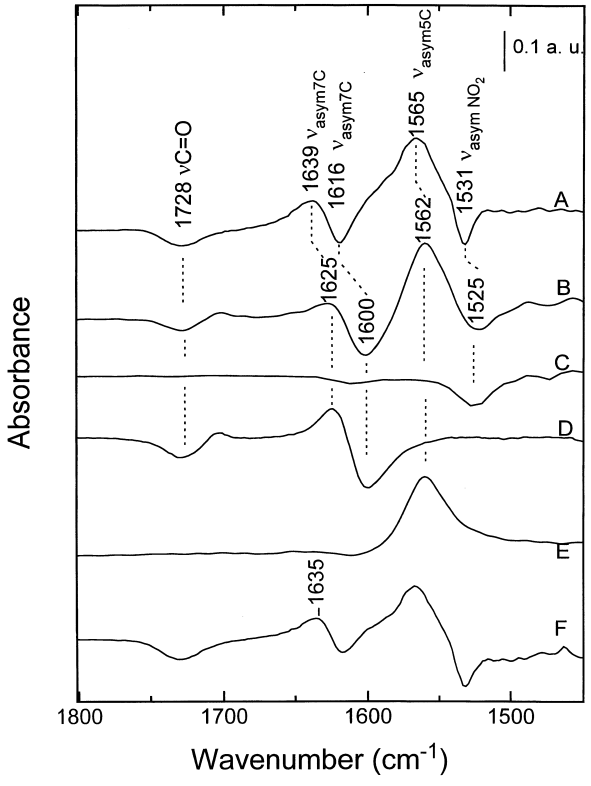

Fig. 2. A: Difference FTIR spectrum between caged glutamate after and before photolysis. B: FTIR spectrum generated by adding the spectra of nitrosobenzene, pyruvate, and acetate, and subtracting the spectra of nitrobenzene and acetoxyacetate. C: Difference FTIR spectrum between nitrosobenzene and nitrobenzene. D: Difference FTIR spectrum between pyruvate and acetoxyacetate. E: FTIR spectrum of sodium acetate. F: Difference FTIR spectrum between caged glutamate after and before photolysis in the presence of 10 mM bisulfite.

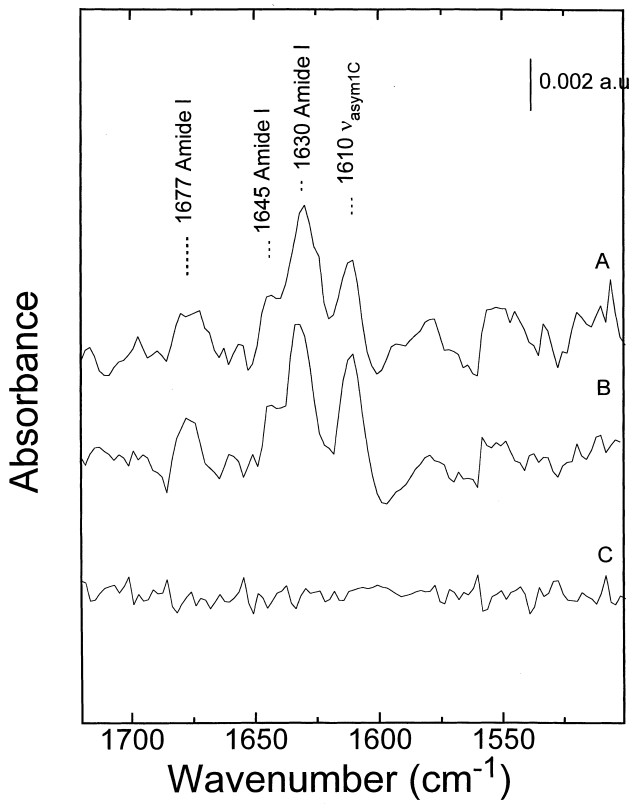

Fig. 3. A: Difference FTIR spectrum between S1S2 protein in D_2O in the presence of caged glutamate after and before photolysis. Features due to excess caged glutamate and photolysis products were subtracted using reference spectra of these compounds. B: Difference FTIR spectrum between S1S2 protein in D_2O in the presence and absence of saturating amounts of free glutamate. Features due to excess glutamate were subtracted using a glutamate reference spectrum. C: FTIR spectrum generated by subtracting the spectrum of S1S2 protein in the presence of caged glutamate with the spectrum of the unbound form of S1S2 protein, and the spectrum of caged glutamate in D_2O.

to the presence of the electron-withdrawing groups nitrobenzene in the caged glutamate and nitrosobenzene in the *o*-nitrosophenyl glyoxalic acid by-product, which increases the frequency of the asymmetric carboxylate vibration [25]. In addition, based on the spectra of pyruvate and acetoxyacetate (individual spectra not shown) the difference feature at 1728 cm^{-1} in the caged glutamate difference spectrum can be assigned to be a composite of the C=O stretching vibration of the *o*-nitrosophenyl glyoxalic acid by-product and the 5C C=O stretching vibration of the caged glutamate. Further, the band at 1565 cm^{-1} in the caged glutamate difference spectrum can be assigned to the 5C asymmetric carboxylate vibration of the photolytically released glutamate, based on the spectrum of sodium acetate (Fig. 2E).

The difference FTIR spectrum of the caged glutamate after and before photolysis was also obtained in the presence of bisulfite (Fig. 2F). It is necessary to use bisulfite with the nitrobenzyl caged compounds when studying protein kinetics, since the by-product *o*-nitrosophenyl glyoxalic acid can react with the sulfhydryl groups of the protein [8,26]. In the presence of bisulfite, this side reaction is quenched, since bisulfite acts as the nucleophile instead of the protein sulfhydryl groups and reacts with the nitroso moiety to form a hydroxyl-

amine derivative [8,26]. The difference spectrum nearly reproduces that seen for the caged compound alone, except for the 4 cm^{-1} downshift in the positive band at 1639 cm^{-1}. The positive feature at 1639 cm^{-1} is assigned to the ν_{asym7C} vibrational mode of the by-product, and the downshift in this mode is suggestive of a decrease in the bond strength at this carboxylate. This decrease in bond strength can be attributed to an internal hydrogen bond between the hydroxylamine moiety and the carboxylate [8]. The reaction of the bisulfite with the nitroso moiety of the *o*-nitrosophenyl glyoxalic acid is not directly observed in the FTIR difference spectrum, since neither the nitroso nor the hydroxylamine groups have any prominent bands in the 1450–1800 cm^{-1} region.

3.2. *Interactions of caged glutamate and its photolysis products with S1S2 protein*

Fig. 3A shows the FTIR difference spectrum of the S1S2 protein in the presence of caged glutamate and bisulfite after and before photolysis. Features due to the excess glutamate, by-products, and caged glutamate were subtracted from the S1S2 difference spectrum using the difference spectrum between the caged glutamate and the photolysis products (Fig. 2F). The difference spectrum thus obtained exhibits changes in the vibrational modes of the protein due to glutamate binding, as well as the vibrational modes due to the bound glutamate (Fig. 3A). The modes arising from the bound glutamate have been assigned using glutamate isotopomers (a detailed analysis of the various bands is provided in [23]). These studies have indicated that the positive band at 1610 cm^{-1} arises from the 1C asymmetric carboxylate vibrations of the bound glutamate. The other dominant features at 1677 cm^{-1}, 1645 cm^{-1}, and 1630 cm^{-1} in the difference spectrum (Fig. 3A), are at frequencies characteristic of amide I modes arising from turns, α-helices, and β-sheet secondary structures, respectively [27]. The positive bands at these frequencies indicate that there is a modest increase in the content of all three ordered secondary structure elements in the protein when it binds to glutamate.

In addition to studying the ligand and protein secondary environments, changes in the tertiary interactions upon binding to the photolytically released glutamate were investigated by studying the environment at the single cysteine (Cys-426) residue in the protein. The environment of this residue was probed by studying the S–H stretching vibration (Fig. 4A). The FTIR difference spectrum indicates that the S–H stretching vibration in the bound form of the protein is downshifted by 15 cm^{-1}, relative to the frequency of this mode in the unbound form. This downshift suggests that the hydrogen bonding at this cysteine is stronger [28,29] in the bound form of the protein relative to the unbound form.

Comparing the difference spectrum between the bound and unbound forms of the S1S2 protein when the glutamate is photolytically released (Figs. 3A and 4A), with the difference spectrum obtained when free glutamate is added (Figs. 3B and 4B), it is evident that the features are identical. Since the spectral features in the S1S2 difference spectrum, as discussed earlier, exhibit signatures characterizing the bound glutamate, the secondary structure of the protein, and tertiary interactions, it can be concluded that the glutamate liberated from the caged form induces the same changes in the S1S2 protein as that of free glutamate and also that the by-products released upon photolysis of the caged glutamate do not interact with the protein, at least for the moieties detected in the spectral range studied.

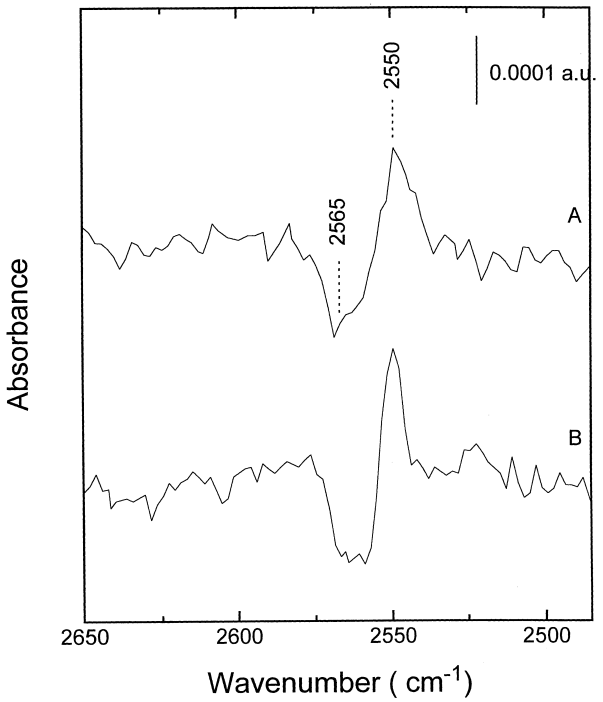

Fig. 4. A: Difference FTIR spectrum between S1S2 protein in water in the presence of caged glutamate after and before photolysis, indicating the changes in the S–H stretching vibration. B: Difference FTIR spectrum between S1S2 protein in the presence and absence of free glutamate, indicating the changes in the S–H stretching vibration.

The inertness of the unphotolysed caged glutamate was also tested by studying the difference FTIR spectrum between S1S2 protein in the presence and absence of caged glutamate. The bands arising due to caged glutamate were removed from the difference spectrum (Fig. 3C), using the caged glutamate spectrum. The subtraction was performed until all the bands arising from the caged glutamate were removed. The difference spectrum thus obtained has no features, thus suggesting that there are no changes induced in the S1S2 protein due to the presence of caged glutamate. Therefore, it can be concluded that the caged glutamate does not interact with the protein and is thus inert for the purposes of FTIR analysis of the kinetics of conformational changes.

The present study, for the first time, provides direct structural evidence that the caged glutamate is inert, and upon photolyis binds to the ligand-binding domain of the glutamate receptor inducing the same structural changes in the protein as when free glutamate is added. Recently, stopped-flow measurements of intrinsic fluorescence demonstrated that agonist binding to wild-type and mutant S1S2 is most likely a two-step process, in which rapid docking to lobe I is followed by a relatively slow isomerization of the protein [22]. Unfortunately, rate constants could not be determined for several reactions that were faster than the limited temporal resolution of the stopped-flow apparatus. Neither temperature- nor pressure-jump experiments are feasible in this system. However, the data presented here suggest that the reaction can be followed at substantially higher temporal resolution using a combination of flash photolysis and FTIR signals. Furthermore, it

may be feasible to follow in parallel the kinetics of individual structural changes and chemically defined binding interactions. Such a study will provide insight into the protein motions in the ligand-binding domain of the glutamate receptor, which control the quaternary changes involved in ion channel formation and desensitization.

References

[1] Marriott, G. (1998) Methods Enzymol. 291.
[2] Hess, G.P. (1996) Arch. Physiol. Biochem. 104, 752–761.
[3] Corrie, J.E.T. and Trentham, D.R., in: Bioorganic Photochemistry (Morrison, J., Ed.), Vol. 2, pp. 243–305, Wiley, New York.
[4] Adams, S.R. and Tsien, R.Y. (1993) Annu. Rev. Physiol. 55, 755–783.
[5] Givens, R.S. (1993) Chem. Rev. 93, 55–66.
[6] McCray, J.A., Herbette, L., Kihara, T. and Trenthan, D.R. (1980) Proc. Natl. Acad. Sci. USA 77, 7231–7241.
[7] Schupp, H., Wong, W.K. and Schnabel, W. (1987) J. Photochem. 36, 85–97.
[8] Barth, A., Corrie, J.E.T., Gradwell, M.J., Maeda, Y., Mantele, W., Meier, T. and Trentham, D.R. (1997) J. Am. Chem. Soc. 119, 4149–4159.
[9] Wieboldt, R., Gee, K.R., Niu, L., Ramesh, D., Carpenter, B.K. and Hess, G.P. (1994) Proc. Natl. Acad. Sci. USA 91, 8752–8756.
[10] Ozawa, S., Kamiya, H. and Tsuzuki, K. (1998) Prog. Neurobiol. 54, 581–618.
[11] Sommer, B. and Seeburg, P.H. (1992) Trends Neurosci. 13, 291–296.
[12] Hollmann, M. and Heinemann, S. (1994) Annu. Rev. Neurosci. 17, 31–108.
[13] Mayer, M.L. and Westbrook, G.L. (1987) Prog. Neurobiol. 28, 197–276.
[14] Torimitsu, K.N.O. (1998) NeuroReport 9, 599–603.
[15] Katz, L.C. and Dalva, M.B. (1994) J. Neurosci. Methods 54, 205–218.
[16] Stern-Bach, Y., Bettler, B., Hartley, M., Sheppard, P.O., O'Hara, P.J. and Heinemann, S.F. (1994) Neuron 13, 1345–1357.
[17] Kuusinen, A., Arvola, M. and Keinänen, K. (1995) EMBO J. 14, 6327–6332.
[18] Lampinen, M., Pentikainen, O., Johnson, M.S. and Keinänen, K. (1998) EMBO J. 17, 4704–4711.
[19] Chen, G.Q. and Gouaux, E. (1997) Proc. Natl. Acad. Sci. USA 94, 13431–13436.
[20] Kuusinen, A., Abele, R., Madden, D.R. and Keinänen, K. (1999) J. Biol. Chem. 274, 28937–28943.
[21] Abele, R., Svergun, D., Keinänen, K., Koch, M.H.J. and Madden, D.R. (1999) Biochemistry 38, 10949–10957.
[22] Abele, R., Keinänen, K. and Madden, D.R. (2000) J. Biol. Chem., in press.
[23] Jayaraman, V., Keesey, R. and Madden, D.R. (2000) Science, submitted.
[24] Madden, D.R., Abele, R., Andersson, A. and Keinänen, K. (2000) Eur. J. Biochem., submitted.
[25] Spinner, E. (1967) J. Chem. Soc. B 874–879.
[26] Kaplan, J.H., Forbush III, B. and Hoffman, J.F. (1978) Biochemistry 17, 1929–1935.
[27] Spiro, T.G. (1987) Raman Spectra and the Conformations of Biological Macromolecules, Wiley, New York.
[28] Li, H. and Thomas Jr., G.J. (1991) J. Am. Chem. Soc. 113, 456–462.
[29] Thomas Jr., G.J. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 1–27.