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Active Site of Bacteriorhodopsin

FTIR and ^{1}H NMR Studies using a Model Molecule

Bogumil Brzezinski and Jerzy Olejnik
Faculty of Chemistry, A. Mickiewicz University, Grunwaldzka 6 PL-60-780 Poznań, Poland
Georg Zundel*
Institute of Physical Chemistry, University of Munich, Theresienstr. 41, D-80333 Munich, Germany

We obtained the 2-*N*-methylaminoethylguanidine amide, **4**, of Kemp's triacid(all-*cis*-1,3,5-trimethylcyclohexane 1,3,5-tricarboxylic acid) as a model substance for the active site of bacteriorhodopsin. Compound **4** was synthesised from Kemp's triacid triethyl ester, **1**, in three reactions. Compound **4** and its complex with tetrabutylammonium 4-methylphenolate were studied by FTIR and ^{1}H NMR spectroscopy in acetonitrile solutions. In the case of compound **4**, two types of hydrogen bond are formed: one is the $CO_2H\cdots N \leftrightharpoons CO_2^-\cdots H^+N$ bond. In this case, the donor is one of the two carboxylic acid groups, and the acceptor, the guanidine group. A double-minimum proton potential is present in this bond and therefore it exhibits large proton polarizability. The second $NH\cdots O{=}C$ hydrogen bond formed between the protonated guanidine (proton donor group) and the carbonyl O atom of the other carboxylic group is asymmetrical. The proton is localised at the guanidine residue. If a phenolate molecule is added to the solution of compound **4**, the situation changes dramatically. A $PhOH\cdots N \leftrightharpoons PhO^-\cdots H^+N$ bond with large proton polarizability is formed between the phenolate and guanidine groups. The polarizable carboxylic acid–guanidine hydrogen bond is broken and the asymmetrical $NH\cdots O$ bond between guanidine and the O atoms of carboxylic acid becomes much stronger. The results obtained with the model are compared with those obtained earlier with bacteriorhodopsin.

It is well known that hydrogen bonds with a double minimum or a broad flat well show very large polarizabilities due to proton shifts .[1–4] This is particularly true for hydrogen-bonded chains in which the protons fluctuate, *i.e.* chains in which collective proton motion occurs. Such hydrogen bonds and hydrogen-bonded systems are indicated by intense continua in the IR spectra. The behaviour of these continua is dependent on the character of the groups involved in these hydrogen bonds and hydrogen-bonded chains.[1–11]

Recently, we studied the FTIR difference spectra of the intermediates of the photocycle of bacteriorhodopsin. In the L_{550} intermediate of bacteriorhodopsin an intense IR continuous absorption was observed, indicating the presence of a proton pathway with large proton polarizability which may conduct protons due to collective proton motion within ps from the active site to the outside of the membrane.[12] According to Henderson *et al.*,[13] in the active site of bacteriorhodopsin the Schiff's base, Asp-85, Arg-82, Tyr-185, Asp-212, Tyr-57, Tyr-79, Tyr-83 and Tyr-64 are present. From ref. 14 and 15 it is known that in the L-550 to M-412 step, one proton transfers from the Schiff's base to Asp-85, and in ref. 16 it is assumed that the Tyr-185 proton shifts to Asp-212. On the basis of our results, model building and the literature data mentioned above, we postulated a mechanism for the proton pumping and suggested a proton pathway.[12]

Experimental

Kemp's triacid was purchased from Aldrich. Kemp's triacid triethyl ester **1** and diethyl esters **2** were obtained following the procedures given in ref. 17 and 18, respectively.

N-Methylaminoethylguanidine Sulfate

To a stirred solution of *S*-methylisothiourea sulfate (Aldrich) (11.10 g, 0.04 mol) in 75 cm^3 of 50% ethanol, 2-*N*-methylaminoethylamine (6.50 g, 0.088 mol) was added during 1 h. At that time a large quantity of methyl mercaptan was evolved. The reaction mixture was then refluxed for an additional hour. The solvents were evaporated under reduced pressure and the residue was recrystallized from an ethanol–ethyl acetate mixture (3 : 2 ratio), yield 3.13 g (48%), mp 210 °C.

N-Methylaminoethylguanidine sulfate was used for the synthesis of the corresponding amide with Kemp's triacid diethyl ester, **3**, following the procedure given in ref. 19. The analytical data of the Kemp's triacid derivatives obtained are given in Table 1.

Table 1 Analytical data of Kemp's triacid and compounds **1–4**,[a] together with the chemical shifts of the CO_2H and NH protons

			analyses			δ	
compound	mp/°C	yield (%)	C (%)	H (%)	N (%)	COOH	NH
Kemp's triacid	241–243	—	—	—	—	12.10	—
1	oil	85	63.09 (63.13)	8.80 (8.83)	—	—	—
2	98	91	60.97 (61.13)	8.27 (8.34)		12.44	—
3	141	45	58.20 (58.23)	8.75 (8.80)	13.61 (13.58)	—	4.74
4	227 dec[b]	85	53.84 (53.92)	7.81 (7.92)	15.81 (15.72)	13.72	7.02
4–phenolate	—	—	—	—	—	17.09	8.27

[a] Calculated data are given in parentheses. [b] Decomposed at this temperature.

The complex of **4** with tetrabutylammonium 4-methyl-phenolate was prepared by adding the respective amounts of 0.1 mol dm^{-3} acetonitrile solutions of tetrabutylammonium phenolate to the solution of **4** in acetonitrile. The solvent was removed under reduced pressure and the solid residue was dissolved in acetonitrile.

The ^1H NMR spectra of compounds **1**–**4** were recorded in [$^2H_3$]acetonitrile (0.1 mol dm^{-3}) at 293 K on a Varian Gemini VT 300 spectrometer using TMS as internal standard.

Spectra of the samples were taken at 293 K in acetonitrile solution (0.1 mol dm^{-3}) with an FTIR spectrometer (Bruker IFS 113v) using a cell with Si windows (sample thickness 0.260 nm, detector DTGS, resolutions 2).

All solvents were stored over 3 Å molecular sieves. All preparations and transfers of solutions were carried out in a carefully dried glove-box under a nitrogen atmosphere.

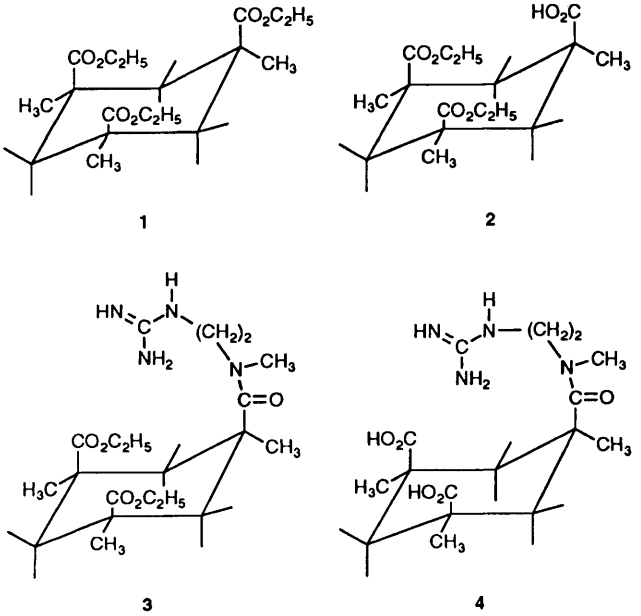
Results and Discussion

It is well known that Kemp's triacid (all-*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid) derivatives are very interesting models for studies of molecular recognition.[20–23] The synthesised compounds are shown in Scheme 1.

The model compounds for the active site of bacteriorhodopsin are compound **4** and its complex with tetrabutylammonium 4-methylphenolate. For comparison we also studied compounds **3** and **1**.

In Table 1 the analytical data of all compounds are given together with their ^1H NMR data. Fig. 1 shows the IR spectra of compound **3**, **4** and of the 1 : 1 complex of **4** with tetrabutylammonium 4-methylphenolate in the region 4000–400 cm^{-1}. The same spectra in the regions 3800–3000, 1800–1500 and 1360–1200 cm^{-1} are shown in Fig. 2–4, respectively.

The spectrum of **3** in Fig. 2 shows the NH_2 and NH stretching vibrations of the guanidine residue at 3491 and 3387 cm^{-1}. In Fig. 3 the ν(C=O) vibration of the $CO_2C_2H_5$ group is observed at 1728 cm^{-1}. Furthermore, the ν(C=O) vibration of the $CONCH_3$ group and the δ(NH) of the guanidine residue are found at 1677 and 1645 cm^{-1}, respectively, *i.e.* in the regions of the amide I and II bands, respectively.



Scheme 1

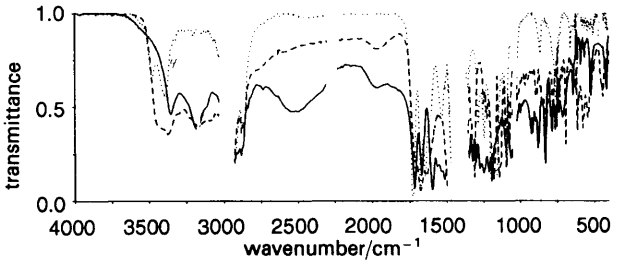


Fig. 1 FTIR spectra of (– – –) compound **4**, (——) its 1 : 1 mixture with 4-methylphenolate and (· · · · ·) compound **3**

No continuous absorption is observed in the spectrum of **3**. In the ^1H NMR spectrum of this compound only one signal due to the guanidine protons is present at 4.74 ppm, *i.e.* at relatively high field.

In the case of compound **4** two intense broad ν(NH) bands are observed at 3453 and at 3371 cm^{-1} (Fig. 2), hence these bands are slightly shifted towards smaller wavenumbers compared with compound **3**. From these bands a continuous absorption extends towards smaller wavenumbers (Fig. 1). This continuum demonstrates that a $CO_2H\cdots N \rightleftharpoons CO_2^-\cdots H^+N$ hydrogen bond is formed. The hydrogen-bond donor is one of the carboxylic acid groups. Note, however, that with regard to both carboxylic acid groups the molecule is symmetrical. The other carboxylic acid group is involved in an asymmetrical $NH\cdots O_2C$ bond, see Scheme 2.

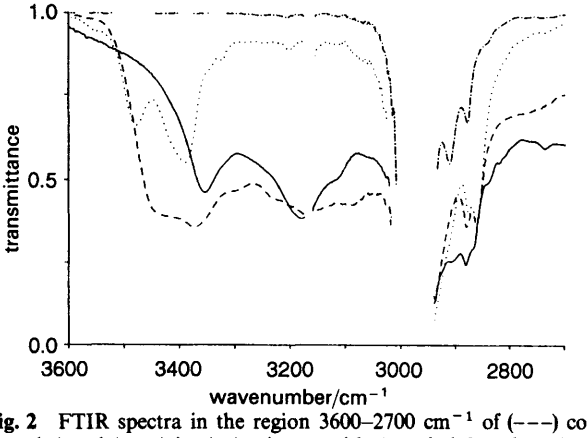


Fig. 2 FTIR spectra in the region 3600–2700 cm^{-1} of (– – –) compound **4** and (——) its 1 : 1 mixture with 4-methylphenolate, (· · · · ·) compound **3** and (– · – · –) compound **1**

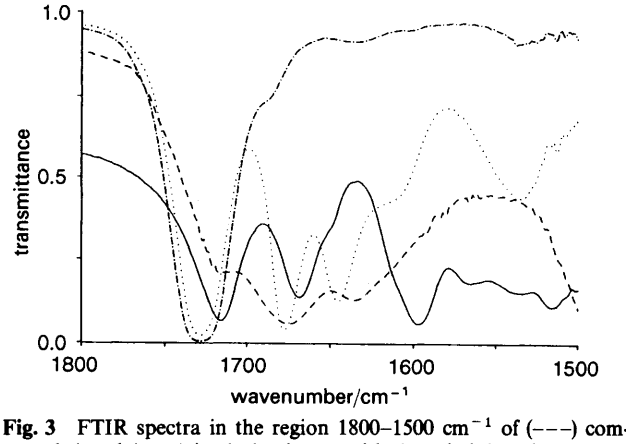


Fig. 3 FTIR spectra in the region 1800–1500 cm^{-1} of (– – –) compound **4** and (——) its 1 : 1 mixture with 4-methylphenolate, (· · · · ·) compound **3** and (– · – · –) compound **1**

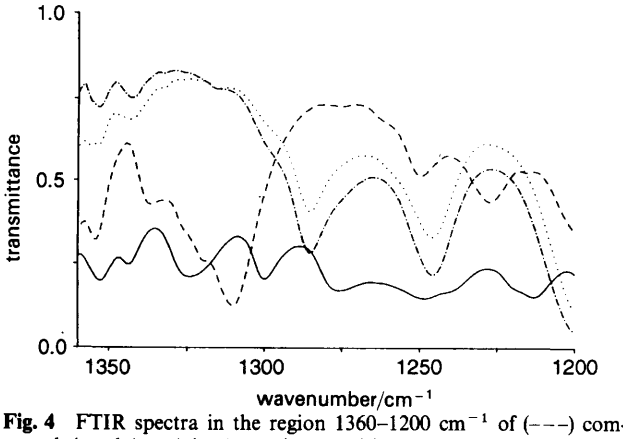
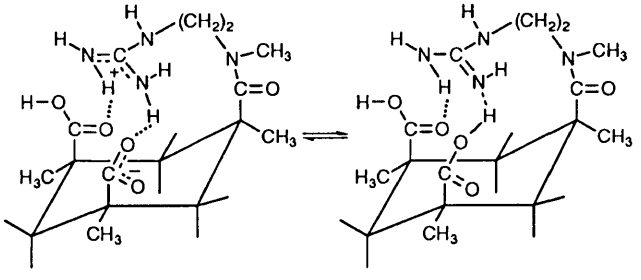
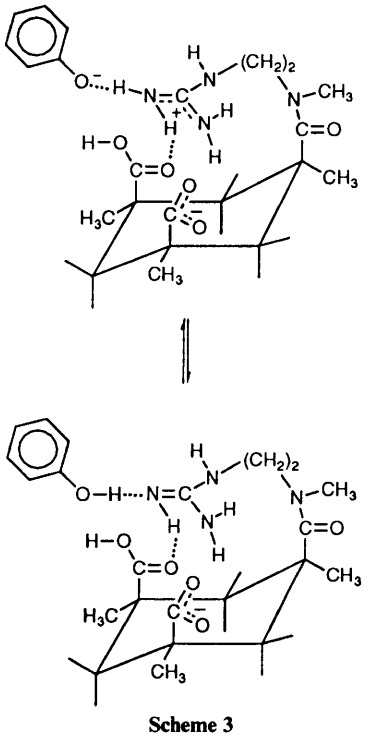


Fig. 4 FTIR spectra in the region 1360–1200 cm^{-1} of (– – –) compound **4** and (——) its 1 : 1 mixture with 4-methylphenolate, (· · · · ·) compound **3** and (– · – · –) compound **1**

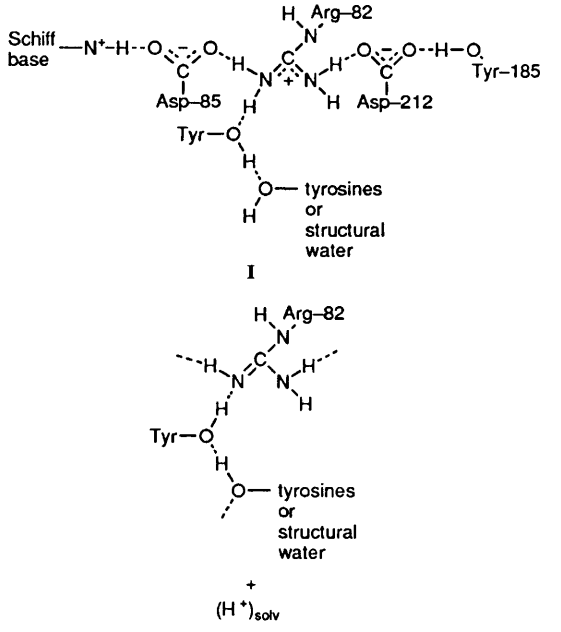
This result is confirmed by the carbonyl bands in Fig. 3. The dashed spectrum shows that with **4** a band is observed as a shoulder at 1718 cm^{-1}. This band is ν(C=O) in the asymmetrical NH···O=C bond. The intense band (Fig. 4) at 1308 cm^{-1} could be the band with ν(C—O) character. The band at 1675 cm^{-1} is caused, as with compound **3**, by the amide I vibration. Finally, the band at 1633 cm^{-1} is the $\nu_{as}(CO_2^-)$ of the CO_2^- group involved in the $CO_2H\cdots N \rightleftharpoons CO_2^-\cdots H^+N$ bond with large proton polarizability. This band is very



Scheme 2



Scheme 3



Scheme 4

strongly broadened due to the fluctuation of the proton. The same is true with the NH bands at 3453 and 3371 cm^{-1} mentioned above. These are slightly shifted towards smaller wavenumbers, intensified and broadened compared with those of **3**, due to the formation of these hydrogen bonds. In the ^1H NMR spectrum (Table 1) of **4**, only one signal for the two CO_2H protons and also only one signal for the NH protons of the guanidine residue are found.

1 : 1 Mixture of 4 with Tetrabutylammonium 4-Methylphenolate

The spectra shown as solid lines in Fig. 1–4 are for 1 : 1 mixtures of **4** with tetrabutylammonium 4-methyl phenolate. The intense broad ν(NH) band at 3453 cm^{-1} has vanished. One ν(NH) vibration is now found at 3353 cm^{-1}. A continuum is observed extending towards smaller wavenumbers. showing a band-like structure in the region 2800–2400 cm^{-1}. This continuum is caused by an $OH\cdots N \rightleftharpoons O^-\cdots H^+N$ bond between phenolate and the guanidine residue, see Scheme 3.

The $CO_2H\cdots N \rightleftharpoons CO_2^-\cdots H^+N$ bond is broken since, owing to the influence of the phenolate group, the NH is a less strong donor. Hence this band is broken although the steric arrangement would favour the formation of this bond. This result is demonstrated by the $\nu_{as}(CO_2^-)$ vibration band, since this band observed at 1633 cm^{-1} with **4** has vanished and a narrower $\nu_{as}(CO_2^-)$ band is found at 1595 cm^{-1}, characteristic of a non-hydrogen-bonded CO_2^- group. The N atom of guanidine becomes a stronger acceptor with the addition of phenolate. Thus, the asymmetrical OH···N bond becomes stronger, as demonstrated by the increase in intensity of the ν(C=O) at 1733 cm^{-1} and the vanishing of the band at 1308 cm^{-1}. However, this hydrogen bond still remains asymmetrical. This result is further confirmed by the pronounced low-field shift of the ^1H signal of the NH protons; this signal is now observed at 17.09 ppm (Table 1). The ν(C—O) vibrations of the phenol and phenolate groups are observed at 1213 and 1241 cm^{-1}, respectively.[24,25] Thus, the complex has to be represented by the formula shown above, containing a $PhOH\cdots N \rightleftharpoons PhO^-\cdots H^+N$ hydrogen bond with large proton polarizability.

Comparison with the Active Centre of Bacteriorhodopsin

In the active centre of bacteriorhodopsin, Arg-82 is probably surrounded by Asp-85, Asp-212 and a tyrosine residue.[12] Scheme 4 shows this arrangement.

Our model demonstrates that the proton fluctuates in a guanidine–phenolate hydrogen bond. The presented model differs, however, from the real system, since the hydrogen-bonded pathway at the tyrosine is missing. We have, however, already shown with another model molecule that an *N*-base–tyrosine–tyrosine hydrogen-bonded system exhibits large proton polarizability[26] and is suitable as a proton pathway.

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