The conformation of an inhibitor bound to the gastric proton pump

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Abstract Substituted imidazo[1,2-*a*]pyridines are pharmaceutically important small molecule inhibitors of the gastric H^+/K^+-ATPase, the membrane-bound therapeutic target for peptic ulcer disease. A non-perturbing analytical technique, rotational resonance NMR spectroscopy, was used to measure a precise (to ±0.2 Å) distance between atomic sites in a substituted imidazo[1,2-*a*]pyridine, TMPIP, bound to H^+/K^+-ATPase at its high-affinity site in the intact, native membrane. The structural analysis of the enzyme–inhibitor complex revealed that the flexible moiety of TMPIP adopts a '*syn*-type' conformation at its site of action. Hence, the conformation of an inhibitor has been resolved directly under near-physiological conditions, providing a sound experimental basis for rational design of many active compounds of pharmaceutical interest. Chemically restraining the flexible moiety of compounds like TMPIP in the *syn*-type binding conformation was found to increase activity by over 2 orders of magnitude. Such information is normally only available after extensive synthesis of related compounds and multiple screening approaches.

Key words: Proton pump; Drug design; Membrane target; Solid state NMR; Peptic ulcer

1. Introduction

Gastric H^+/K^+-ATPase, an αβ heterodimer with a unit molecular weight of over 150 kDa, maintains the highly acidic milieu of gastric glands by functioning as a nucleotide-driven proton pump within the apical membrane of stimulated parietal cells [1]. The gastric proton pump plays an important role in the pathology of peptic ulcer disease and is a well-established therapeutic target [2]. Proton pump inhibitors are widely recognized as important drug candidates [3] as testified by the high share of the pharmaceutical market held by the anti-ulcer drug, omeprazole. Determining the three-dimensional structure of inhibitors at the active site of a therapeutic target like the gastric proton pump can provide a rationale for the design of novel drugs [4,5]. Structure-based design can reduce significantly the labour required for drug discovery compared with random screening. Unfortunately, determining structures of drugs bound to proteins that function within the cell membrane is a formidable challenge, largely because of the difficulty of obtaining crystals for X-ray diffraction. Consequently, no high-resolution structural information is available for drugs bound to membrane proteins and a structure-based design strategy has yet to be realised.

We aimed to obtain direct information on the structure of a substituted imidazo[1,2-*a*]pyridine (SIP), a potent inhibitor of gastric H^+/K^+-ATPase, when bound to its site of action in the native gastric membrane, under conditions that retained the structural and functional integrity of the enzyme. Much is known about the binding characteristics of such pharmaceutically important SIPs and their structure–activity relationship (Table 1). An aromatic group at the 8-position of the imidazopyridine ring is an essential requirement for activity, as is the orientation of the aromatic group relative to the fused ring [6]. TMPIP (Table 1; compound IV) is structurally one of the most simple of the active SIPs. The entire conformational flexibility of TMPIP is localized around the aromatic 8-phenylmethoxy substituent, and is defined by three torsional angles, ϕ_1, ϕ_2 and ϕ_3 (Table 1). Whereas ϕ_2 and ϕ_3 cannot be determined unambiguously from a single internuclear distance, ϕ_1 is a direct function of the distance r_{IS} between the atomic carbon sites C10 and C14 (Fig. 1).

2. Methods and results

Rotational resonance nuclear magnetic resonance (RRNMR) spectroscopy [7]was used here to determine the structurally diagnostic distance r_{IS}, and hence the angle φ_1, for TMPIP bound to the gastric proton pump. Techniques such as RRNMR have provided structural details for membrane proteins in two other cases where crystallography is not possible [8,9] and for lyophilized water-soluble ligand–protein complexes [10,11], but never for a ligand in a receptor. RRNMR enables precise measurement of distances of up to 7 Å between pairs of certain atoms (such as ^{13}C), introduced at specific molecular sites as non-perturbing probes [8]. Here, two carbon sites of TMPIP (C10 and C14) were enriched in ^{13}C to enable the distance r_{IS}, to be measured using RRNMR.

Prior to carrying out structural analysis of the inhibitor–proton pump complex, initial NMR experiments were performed to establish the conditions required for detecting the bound inhibitor selectively [12]. NMR spectra were obtained from porcine H^+/K^+-ATPase membranes after titrating in TMPIP, under conditions that assimilated the functional, physiological environment of the protein. All spectra of TMPIP in the gastric membrane suspension were processed to eliminate interfering signal from lipids and protein within the membrane. Two peaks remained in the spectrum after processing, which corresponded to the labelled C10 and C14 sites of TMPIP (Fig. 2A). The experiment detected only TMPIP bound to the proton pump, while unbound inhibitor was invisible [12].

Photoaffinity labelling of the H^+/K^--ATPase with a radiolabelled diazonium derivative of TMPIP (Table 1; compound III) indicated that the inhibitor binds predominantly to a single high-affinity site [13]. To ascertain whether the NMR spectra represented TMPIP at this single site or at additional locations, the H^+/K^+-ATPase was titrated with KCl, which competes out TMPIP only from the high-

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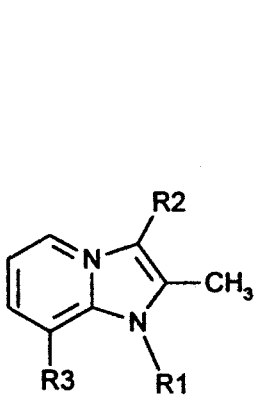
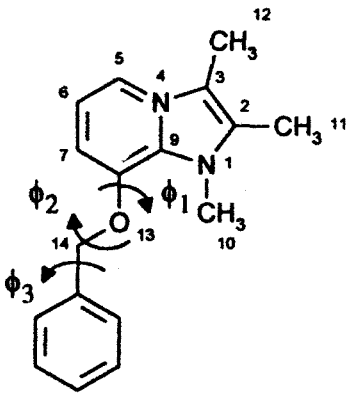
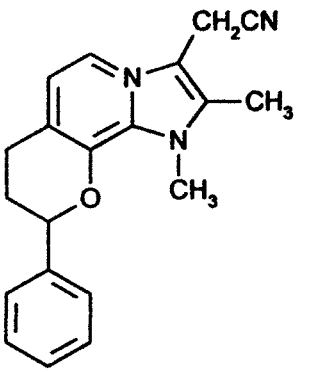
Table 1
Summary of the binding and activity of some substituted imidazo[1,2-*a*]pyridines



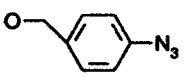
I-III



IV



V

Compound	R1	R2	R3	$IC_{50}(K_i)$
I	H	CH_2CN	OCH_2Ph	1.6 μM[a] (35 nM)[b]
II	CH_3	CH_2CN	OCH_2Ph	50 nM (14 nM)[b]
III	CH_3	CH_3		3.7 μM[b] (2.0 mM)[b]
IV		See above		4.0 mM[c]
V		See above		0.1 mM[a]

Activity was measured as inhibition of K^+ stimulated ATP hydrolysis by H^+/K^+-ATPase. Activity and binding kinetics were determined at 37°C on porcine H^+/K^+-ATPase purified in lyophilized gastric microsomes. In cases where one or more of the source references report different results, the highest value is stated here. The chemical structure, numbering system and definition of torsional angles (ϕ_1, ϕ_2, ϕ_3) are illustrated for compound IV, the 1,2,3-trimethyl-8-(phenylmethoxy)-imidazo[1,2-*a*]pyridinium cation (TMPIP).[a]Ref. [6]. [b]Ref. [14]. [c]Determined here.

affinity site. In this experiment, no signal from bound TMPIP was detected in the NMR spectrum (Fig. 2B) confirming that, in the absence of KCl, the NMR spectra of the inhibitor–proton pump complex represent TMPIP bound wholly to the single active site of interest.

Having ascertained that bound TMPIP can be detected selectively to give exact knowledge of its binding location, a series of RRNMR spectra were obtained for the inhibitor–proton pump complex (Fig. 2C). Reference spectra were also acquired at a spinning frequency well off rotational resonance. The data points (Fig. 3) indicated a decay of difference magnetization on rotational resonance (RR), whereas off RR the exchange process was quenched. The numerical simulations providing the theoretical curves [7] took into account the uncertainties in some of the simulation parameters. The experimental data fell within theoretical curves corresponding to a distance within the range 3.9 Å $< r_{IS} <$ 4.3 Å, which translated to a torsional angle φ_1 of between 0° and 42°. Hence, the experimental data indicated that TMPIP favours a '*syn*-type' configuration with respect to φ_1 when bound to H^+/K^+-ATPase at the high-affinity site (Fig. 3C; right).

3. Discussion

A series of substituted imidazo[1,2-*a*]pyridines have been synthesized in which the flexibility of the aromatic 8-substituent was chemically restrained within a limited range of conformations [6]. Inhibitory potency of these compounds was highly dependent on the restricted conformation of the aromatic group [6]. It is notable that the most active compound (Table 1; compound V) was constrained about the same *syn* configuration found here for bound TMPIP. Clearly, the structure–activity relationship (SAR) of SIPs was hitherto only available from systematic chemical synthesis. Here, the recognized docking conformation of these inhibitors was determined directly in a single experiment.

In conclusion, it has been possible to provide specific and precise (to ±0.2 Å) structural information for a small molecule bound specifically to a large integral membrane protein under near-physiological conditions. Although in this case our findings benefit from retrospection of available SAR information obtained by systematic medicinal chemistry, it will be possible in future work to examine the binding conformation of H^+/K^+-ATPase inhibitors for which prior structural data are not available.

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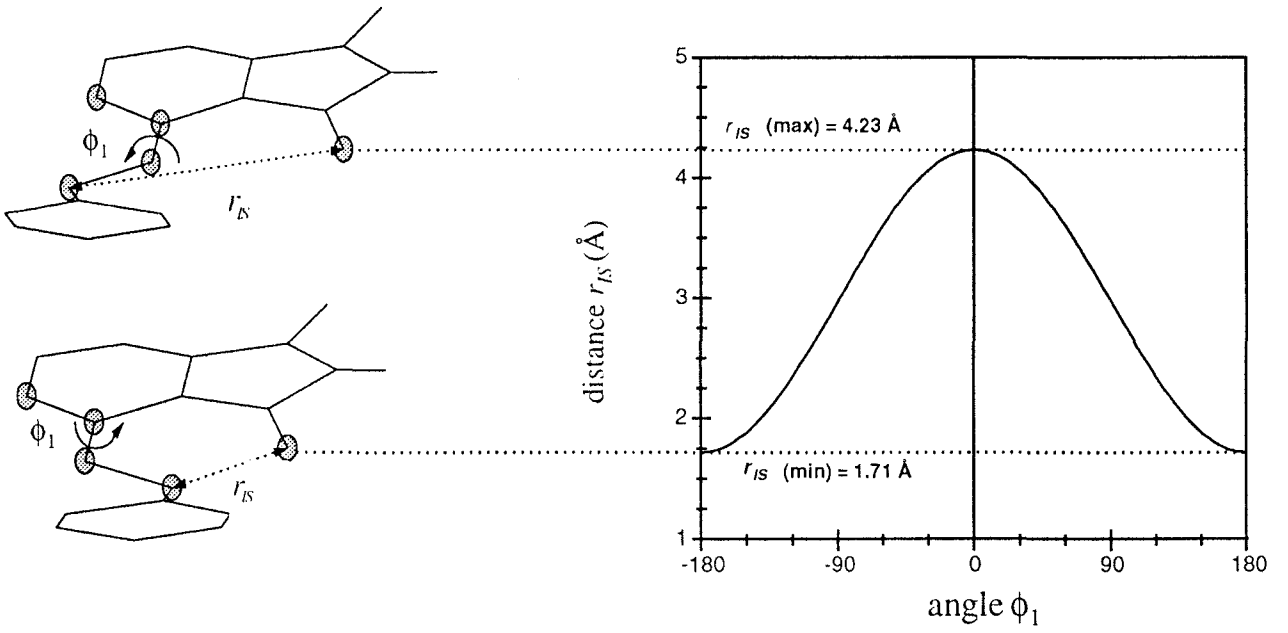

Fig. 1. The interrelationship of distance r_{IS}, torsional angle ϕ_1 and molecular conformation of TMPIP.

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References

[1] Forte, J.G. et al. (1980) Annu. Rev. Physiol. 42, 111.
[2] Sachs, G. (1988) Annu. Rev. Pharmacol. Toxicol. 28, 269.
[3] Pope, A.J. and Sachs, G. (1992) Biochem. Soc. Trans. 20, 566.
[4] Alberg, D.G. and Schreiber, S.L. (1993) Science 262, 248.
[5] von Itzstein, M. et al. (1993) Nature 363, 418.
[6] Kaminski, J.J. et al. (1991) J. Med. Chem. 34, 533.
[7] Levitt, M.H. et al. (1990) J. Chem. Phys. 92, 6347.
[8] Creuzet, F. et al. (1991) Science 251, 783.
[9] Watts, A. et al. (1995) Mol. Membr. Biol. 12, 233.
[10] Holl, S.M. et al. (1992) J. Am. Chem. Soc. 114, 4830.
[11] McDowell, L.M. et al. (1995) J. Am. Chem. Soc. 117, 12352.
[12] Spooner, P.J. et al. (1994) Proc. Natl. Acad. Sci. USA 91, 3877.
[13] Munson, K.B. et al. (1991) J. Biol. Chem. 266, 18976.
[14] Keeling, D.J. et al. (1989) J. Biol. Chem. 10, 5552.

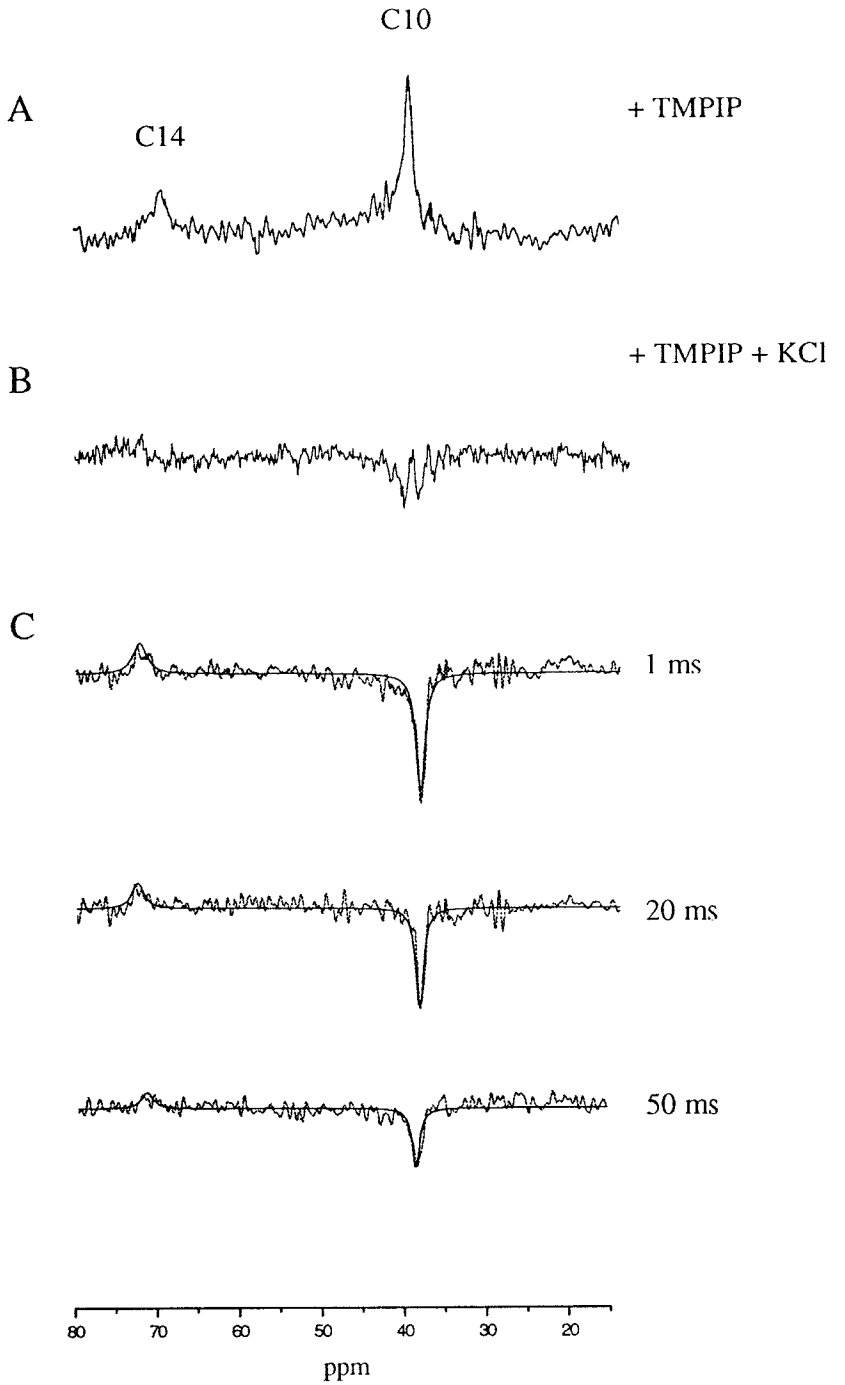
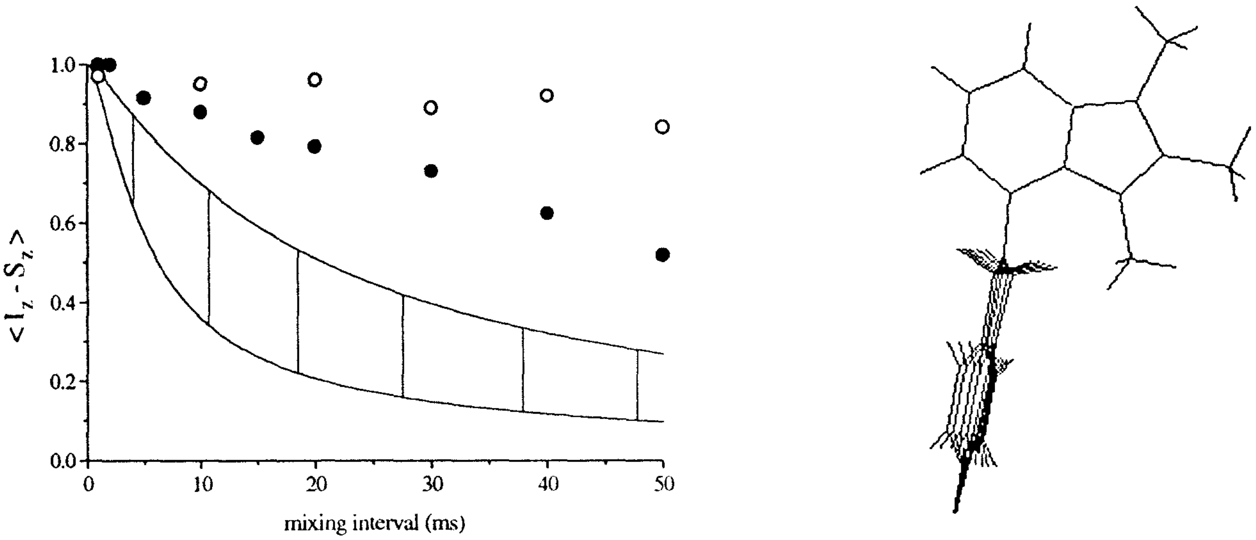


Fig. 2. A series of ^{13}C NMR spectra (at 9.3 T) of H^+/K^+-ATPase-enriched gastric membranes containing TMPIP. All spectra were recorded at 1°C. The membranes were prepared as a hydrated pellet from porcine fundic mucosal cells, by differential and density gradient centrifugation [13]. Cross-polarization magic angle spinning (CPMAS) difference spectroscopy was used to detect signals only from TMPIP bound to the proton pump [12]. (A) Spectrum of bound TMPIP, showing peaks at 37 and 71 ppm from C10 and C14, respectively. (B) Spectrum after incubation of membranes with TMPIP and KCl. K^+ ions compete with SIPs for the high-affinity site of the H^+/K^+-ATPase during the phosphoenzyme stage of the enzyme cycle [14]. A large excess of KCl prevents TMPIP binding to its inhibitory site, but not to other non-specific sites. (C) Rotational resonance spectra of the proton pump–TMPIP complex (sample spinning frequency ν_r=3013 Hz; i.e., n=1 RR) at three mixing intervals: 1 ms, 20 ms, 50 ms. On rotational resonance (RR), nuclear magnetization is exchanged between the two carbon sites C10 and C14. The exchange is monitored by creating opposite polarizations of the two spin sites, observed as an inversion of one of the peaks in the NMR spectrum, and following the decay of magnetization over a set of mixing intervals.


1.0
0.8
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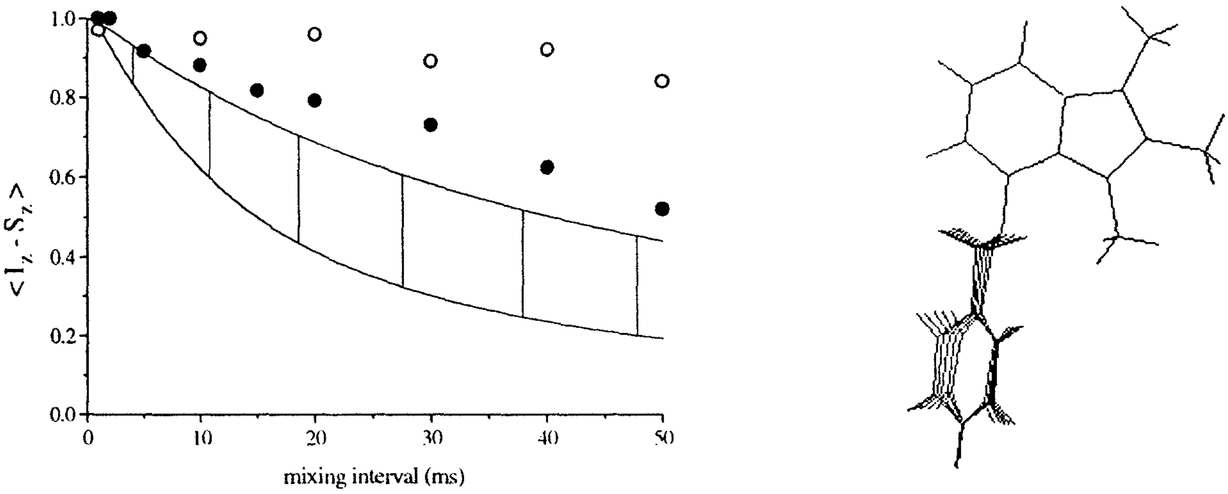
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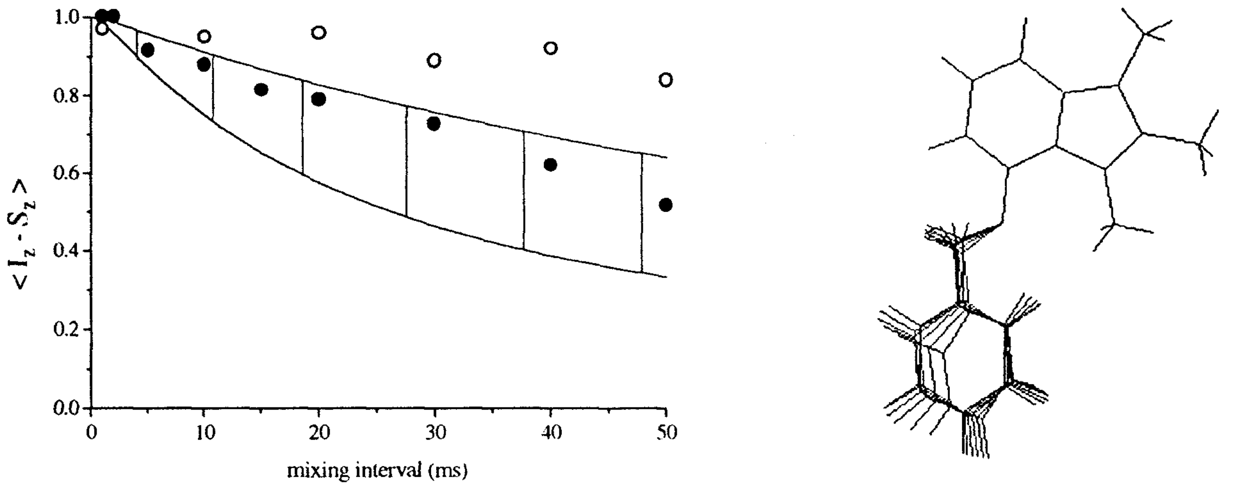
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mixing interval (ms)

Fig. 3. Experimental measurements of magnetization exchange for the proton pump–TMPIP complex at $n = 1$ RR [●] and off RR ($\nu_r = 2500$ Hz [□]). Simulations of magnetization exchange curves are shown for three interatomic distance ranges r_{IS} (solid lines). These curves are the limits defined by the random sampling of a set of internuclear distances as well as shielding tensor orientations [6]. Curves correspond to distance ranges of 3.0–3.4 Å (top graph), 3.5–3.8 Å (middle graph) and 3.9–4.3 Å (bottom graph), where the upper curve corresponds to the longer distance in each case. Molecular conformations of TMPIP corresponding to each distance range are shown alongside each graph. The conformational space traversed by ϕ_1 reflects the r_{IS} ranges bounded by each pair of curves, whereas ϕ_2 and ϕ_3 were obtained by minimum energy calculations. The true distance r_{IS} is derived from the theoretical curves bounding the experimental data [7].
←