Molecular Dynamics Study of the $M_{412}$ Intermediate of Bacteriorhodopsin

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ABSTRACT Molecular dynamics simulations have been carried out to study the $M_{412}$ intermediate of bacteriorhodopsin’s (bR) photocycle. The simulations start from two simulated structures for the $L_{550}$ intermediate of the photocycle, one involving a 13-cis retinal with strong torsions, the other a 13,14-dicis retinal, from which the $M_{412}$ intermediate is initiated through proton transfer to Asp-85. The simulations are based on a refined structure of bR$_{568}$ obtained through all-atom molecular dynamics simulations and placement of 16 waters inside the protein. The structures of the L$_{550}$ intermediates were obtained through simulated photoisomerization and subsequent molecular dynamics, and simulated annealing. Our simulations reveal that the $M_{412}$ intermediate actually comprises a series of conformations involving 1) a motion of retinal; 2) protein conformational changes; and 3) diffusion and reconfiguration of water in the space between the retinal Schiff base nitrogen and the Asp-96 side group. (1) turns the retinal Schiff base nitrogen from an early orientation toward Asp-85 to a late orientation toward Asp-96; (2) disconnects the hydrogen bond network between retinal and Asp-85 and tilts the helix F of bR, enlarging bR’s cytoplasmic channel; (3) adds two water molecules to the three water molecules existing in the cytoplasmic channel at the bR$_{568}$ stage and forms a proton conduction pathway. The conformational change (2) of the protein involves a 60° bent of the cytoplasmic side of helix F and is induced through a break of a hydrogen bond between Tyr-185 and a water-side group complex in the counterion region.

INTRODUCTION

Bacteriorhodopsin (bR) is a membrane protein that functions as a light-driven proton pump in the cell membrane of *Halobacterium halobium*. The function is achieved through a cyclic process initiated by the absorption of a photon. The pump cycle is characterized through a series of intermediates $J_{625}$, $K_{590}$, $L_{550}$, $M_{412}$, $N_{520}$, and $O_{640}$, where the subscripts denote the wavelengths of the respective absorption maxima (Lozier et al., 1975).

A major role in the proton pump cycle of bR$_{568}$ is played by its retinal chromophore, which is bound through a Schiff base linkage to the Lys-216 residue. Retinal undergoes an initial electronic excitation and photochemical transformation. It also acts as a switch, donating a proton to the extracellular side of bR$_{568}$ and accepting a proton from the intracellular side. Retinal and the numbering adopted for its atoms and dihedral angles are presented in Fig. 1.

In bR$_{568}$, retinal is in an all-trans configuration as shown in Fig. 1. A photon triggers an isomerization of retinal and leads to the transition bR$_{568} \rightarrow J_{625}$. The intermediate J$_{625}$ decays thermally to $K_{590}$ and then relaxes to L$_{550}$, at which point retinal is in a 13-cis state (Doig et al., 1991). Possible torsions of bonds other than the 13–14 bond of retinal (Fig. 1) were suggested, as discussed below. During the L$_{550} \rightarrow M_{412}$ transition, retinal’s Schiff base proton is transferred to Asp-85 (Mogi et al., 1989; Braiman et al., 1988; Gerwert et al., 1989; Metz et al., 1992) to be released eventually to the extracelluar space. Retinal remains in a 13-cis isomeric state in the $M_{412}$ intermediate (Pettei et al., 1977; Aton et al., 1977). During the subsequent transition $M_{412} \rightarrow N_{520}$, a proton is transferred to retinal’s Schiff base from Asp-96; the latter side group eventually takes up a proton from the cytoplasmic environment (Gerwert et al., 1989). The photocycle is completed as retinal returns to an all-trans isomeric state and the protein returns to bR$_{568}$, either through the O$_{640}$ intermediate or directly from N$_{520}$ (Váró et al., 1990).

The $M_{412}$ intermediate serves as a switch between a proton release pathway and a protein uptake pathway (Nagle and Tristram-Nagle, 1983; Braiman et al., 1988; Papadopoulos et al., 1990; Gerwert, 1992), the switch disconnecting retinal from the extracellular side and connecting it to the cytoplasmic side (Orlandi and Schulten, 1979; Váró and Lanyi, 1990, 1991a, 1991b). The processes underlying this switch are not known, and it is fair to claim that the elucidation of these processes holds the key to the eventual discovery of the mechanism of bR.

A key difference between explanations of bR’s proton pump mechanism lies in the separate roles of retinal and the protein in realizing the switch function at the $M_{412}$ stage. According to Fodor et al. (1988), the switch is realized through a protein conformational change, with retinal remaining in the same 13-cis geometry in the L$_{550}$ as well as in the $M_{412}$ intermediate. Another model (Schulten and Tavan, 1978; Schulten, 1978; Schulten et al., 1984; Gerwert and Siebert, 1986) suggests that retinal is in a 13,14-dicis configuration in the K$_{590}$ and L$_{550}$ intermediates, and that it...
FIGURE 1 Numbering of carbon atoms and dihedral angles of retinal and the Lys-216 aliphatic chain. The numbers in Roman represent the numbering of the carbon atoms of retinal; the numbers in bold italic represent the numbering of dihedral angles.

functions as a switch through a torsional motion around the 14–15 bond in the M_{412} intermediate.

The arrangement of the retinal chromophore and of key amino acid side groups, as reported in Henderson et al. (1990), supports the notion of a proton channel controlled by retinal. Unfortunately, the low resolution structure in Henderson et al. (1990) does not reveal water, which is expected to play a crucial role in proton conduction. Protein structural transformations amounting to a widening of the cytoplasmic channel have been observed for the M_{412} intermediate (Dencher et al., 1992; Subramanian et al., 1993; Hauss et al., 1994) and should be invoked in an explanation of the switch function of this intermediate. However, the available structural information does not yield an explanation of the pump mechanism, in particular, of the switch mechanism of M_{412}.

Molecular dynamics simulations appear to be suited to provide the missing information, i.e., to place the unresolved water, initiate the phototransformation, and follow the intermediates of the pump cycle up to the M_{412} stage. But such procedures are methodologically extremely demanding, if possible at all, due to several factors: 1) the low resolution structure in Henderson et al. (1990) may contain serious, i.e., misleading, errors; 2) placement of waters by means of computer modeling is difficult; 3) reliable potential surfaces of retinal in the excited state do not exist and, hence, the phototransformation can only be described through ad hoc models, which induce the observed all-trans → 13-cis transformation of retinal; 4) the pump cycle up to the formation of M_{412} requires a time span of microseconds, i.e., a time period too long for conventional molecular dynamics descriptions, but possibly amenable to the simulated annealing method. The difficulties of molecular dynamics descriptions in general, e.g., the limited reliability of the force fields used, and the shortcomings (1–4) in particular, make modeling of the M_{412} intermediate necessarily a highly speculative effort. However, the previous studies of the intermediates up to the L_{550} stage (Humphrey et al., 1994; Humphrey et al., 1995), as outlined below, consistently lead to a small number of candidate structures; the two most pertinent of these structures, referred to as L_{1} and L_{2}, will be investigated in the present study. The results imply that one of the structures, namely L_{1}, leads to an M_{412} intermediate that is in excellent accord with observations and extremely interesting in that the long standing dispute between the above mentioned models for the M_{412} switch function is resolved. Our study suggests that key single bond torsions of retinal, internal waters, and a protein conformational change cooperatively contribute to the switch function of the M_{412} intermediate.

Several molecular dynamics calculations were carried out to shed light on the pump mechanism of bR (Warshel et al., 1991; Bashford and Gerwert, 1992; Scharnagl et al., 1994). One of the early simulations (Zhou et al., 1993) attempted to describe the whole pump cycle by forcing proton transfer reactions to occur on a time scale covered by molecular dynamics simulations, i.e., on a time scale of 100 ps, rather than on the observed millisecond time scale. The simulations revealed that retinal at the M_{412} stage of bR, i.e., after transfer of the Schiff base proton, can act as a proton switch in that the Schiff base nitrogen turns spontaneously from an orientation toward Asp-85 (the proton acceptor) to an orientation toward Asp-96 (the proton donor) after the retinal → Asp-85 proton transfer.

Based on a refined structure of bR_{668} (Humphrey et al., 1994), simulations have been carried out to study the early intermediates L_{625}, K_{590}, and L_{550} (Humphrey et al., 1995). The simulations, sampling different initial conditions, yield a distribution of reaction products falling into four distinct classes: class I, all-trans retinal; class II, 13-cis retinal with the Schiff base proton oriented toward Asp-96; class III, 13-cis with the Schiff base proton oriented perpendicular to the membrane normal (see Fig. 2 (L_{1})); and class IV, 13,14-dicis (see Fig. 2 (L_{2})). To reach the microsecond time scale of formation of the L_{550} intermediate, the study in (Humphrey et al., 1995) employed simulated annealing. The simulations in Humphrey et al. (1995) identified two likely structures for the L_{550} intermediate: one, referred to as L_{1} below, involves a 13-cis retinal with a strong torsion; the other, referred to as L_{2} below, involves a pure 13,14-dicis retinal.
The structure of $L_1$ is presented in Fig. 3. The figure shows the three regions of the proton pathway in bR: 1) the cytoplasmic channel, the space between Asp-96 and the Schiff base including the amino acid side groups Thr-46, Leu-93 and Phe-219; 2) the counterion region, the space near the retinal Schiff base and its counterions including Asp-85, Tyr-57, Tyr-185 and Asp-212; 3) the extracellular channel, the channel between the counterions of the Schiff base and the extracellular side of bR, including the regions around Arg-82, Glu-204 and Glu-9.

In the present paper we employed the $L_{550}$ structures described in Humphrey et al. (1995) to initiate the $M_{412}$ intermediate through transfer of the retinal proton to Asp-85. The following sections describe the methods employed, the results obtained, comparisons with observations, and the emerging explanation of the mechanism of the light-driven proton pump realized by bacteriorhodopsin.

**METHODS**

The simulations and analyses described in this paper were carried out using the program X-PLOR (Brünger, 1992) together with the CHARMM force field (Brooks et al., 1983). A cut-off distance of 8 Å for non-bonded interactions and a dielectric constant $\varepsilon = 1$ were employed. An integration timestep of 1 fs was chosen. Like the simulations of $bR_{460}$ reported in Humphrey et al. (1994), and of the $L_{423}$, $K_{590}$, $L_{550}$ intermediates reported in Humphrey et al. (1995), we employed an all atom model and an explicit hydrogen bonding term in the energy function, both of which are particularly important for the placement of water inside bR.

Following Humphrey et al. (1994) and Humphrey et al. (1995), the protonation states of titratable groups adopted were standard except for Asp-85, Asp-96, and Asp-115, which were assumed to be protonated according to observations reported in Gerwert et al. (1989) and Engelhard et al. (1990). The force field parameters and charges used for bR were, respectively, the parsmallh3.pro parameters and topallhbox.pro charges, except for retinal. For the deprotonated and the protonated Schiff base retinal in bR, we adopted the charges suggested in Zhou et al. (1993), except for changes to fit the explicit hydrogen model, i.e., the explicit hydrogen atoms added to the retinal backbone were given partial charges of 0.03, with the corresponding heavy atom charges reduced by the same amount. The force field parameters for the protonated Schiff base retinal were those used in Humphrey et al. (1994) (1995). The unprotonated Schiff base retinal is assumed to have the same force field parameters as those of the protonated one except that the 13–14, 14–15 and 15–N torsion force constants were 30, 5, and 30 Kcal/mol, respectively, as suggested in Zhou et al. (1993).

Water was placed in the interior of the protein to simulate photocycles in early studies (Warshel et al., 1991; Zhou et al., 1993; Humphrey et al., 1994; Scharnagl et al., 1994). In the present study, 16 water molecules were placed inside bR in the structures of $L_{550}$ (see Fig. 3) (Humphrey et al., 1995). Seven water molecules were placed in the counterion region, three in the cytoplasmic channel, and six in the extracellular channel. These water molecules were described by a TIP3P potential (Jorgensen et al., 1983) with the CHARMM parameter file param19.sol. The 16 water molecules, however, do not include all the possible waters in bR; in placing these waters we focused on the retinal binding site, on the cytoplasmic channel, and on the extracellular channel where we filled all voids that were sufficiently hydrophilic as judged, e.g., by the stability of the water positions. Voids in other parts of bR were disregarded for water placement because we considered respective waters to be less relevant to the photocycle (Zhou et al., 1993; Humphrey et al., 1994).

**Simulated annealing**

The times for the formation and decay of the $M_{412}$ intermediate are in the μs and ms range; presently, computational resources allow one to cover time periods of only a few nanoseconds in molecular dynamics simulations. For a description of the $M_{412}$ intermediate we resorted, therefore, to simulated annealing (van Laarhoven and Aarts, 1987; Brünger et al., 1990; Brünger, 1991).

Annealing involves coupling of the simulated system to a heat bath that is kept at a prescribed temperature and is modulated during the simulation. The bath temperature follows a certain schedule of heating and cooling. Temperatures $T \approx 600$ K adversely affected the structure of bR. Wishing to employ the highest possible temperatures to accelerate the reaction dynamics of our system, we accepted 500 K as the highest annealing temperature because this temperature left the overall structure of bR intact for sufficiently long times, but produced rapid local conformational changes.

For the conventional choice of a barrier of 5 kcal/mol for torsion around the single bonds of retinal, particularly around its 8–9 and 10–11 bonds, the high temperatures chosen in the annealing schedule render the chromophore too flexible. To prevent unrealistic torsions, we constrained all single bond dihedral angles along retinal’s backbone by raising their barriers to 10 kcal/mol during the simulated annealing steps. The SHAKE algorithm for constraining bond lengths was used for all temperatures elevated above 300 K to keep the numerical integration stable for a 1-fs time step. The high torsional barriers and the SHAKE constraints were released during the 300 K equilibration phases of the simulations.

**Replacement of water**

During the photocycle, the conformation of bR changes. We suggest that this change allows water molecules to diffuse into the cytoplasmic channel.
of bR. To mimic the process of additional waters entering the cytoplasmic channel, we removed water molecules from the exit of the extracellular channel and moved the channel to the desired position. Since most structural transitions at the M₄₁₂ stage occur in the cytoplasmic channel and in the counterion region, the removal of water from the far extracellular region is unlikely to affect the dynamics at the M₄₁₂ stage.

### Summary of simulations

As explained above, our simulations started from two structures suggested in Humphrey et al. (1995) for the L₅₅₀ intermediate, L₁, and L₂. Our simulations transferred a proton from the retinal Schiff base to Asp-85 and carried out energy minimization, molecular dynamics simulations, and annealing. The structures obtained from L₁ and L₂ after proton transfer and equilibration will be referred to as M₄ and M₁₄₄, respectively. Structures obtained after further simulations will be similarly denoted as M₄₅, M₆₄₅, etc. and M₁₄₅, M₆₄₅, etc. The simulations to generate later stages of the respective M₄₁₂ intermediates are summarized and defined in Fig. 4.

### RESULTS

As mentioned above, in our simulations we have investigated two models for the M₄₁₂ intermediate, one starting from the L₁ candidate structure for the L₅₅₀ intermediate shown in Fig. 3 and one starting from the L₂ structure. During the M₄₁₂ stage in both models, bR undergoes a series of transformations induced by simulated annealing and equilibration steps summarized in Fig. 4. The induced transformations include structural rearrangements of retinal, alterations in the helices of bR as well as a rearrangement of waters and their hydrogen bond network.

#### Simulation of an M₄₁₂ intermediate starting from L₁

We will consider first the evolution of bR in the case that the M₄₁₂ stage is initiated through a retinal → Asp-85 proton transfer of L₁ (see Fig. 3 and the subsequent simulations summarized in Fig. 4(a)).

#### The M₈ state

The state of bR, obtained following the Schiff base proton transfer of L₁ to Asp-85 and subsequent equilibration, is referred to as the M₈ state. Fig. 5 compares the geometry of retinal in this state with the retinal geometry in bR₅₆₈. For M₈ the retinal backbone from C₅ to C₁₃ (the numbering of retinal’s carbons is defined in Fig. 1) is tilted significantly, and the C₂₀ methyl group is shifted by 1.68 Å toward the cytoplasmic side relative to its position in bR₅₆₈. The retinal structure is in agreement with the report by Hauss et al. (1994), which shows that in M₄₁₂ the polyene chain tilts out of the plane of the membrane toward the cytoplasm by ~11° ± 6°. The 1.68 Å shift is also in agreement with observations reported by Heyn and Otto (1992), which showed that the bR₅₆₈ → M₄₁₂ transformation tilts the transition dipole moment of retinal out of the plane of the membrane and moves the C₂₀ methyl group by 1.7 Å toward the cytoplasmic side of the membrane.

Fig. 6 presents the dihedral angles of retinal in the M₈ state. Significant deviations of the angles from the equilibrium values exist, e.g., the dihedral angle of the 14–15 bond assumes a value of 201.4°. Functionally, the most significant feature of the retinal structure at the M₈ stage is the orientation of the Schiff base and its two neighboring carbon atoms in a direction nearly parallel to the membrane plane. Such conformation of retinal is accommodated by a strained Lys-216 side chain, the dihedral angles of which are shown in Fig. 7.

The configuration of waters, amino acid side groups, and hydrogen bonds of M₈ is presented in Fig. 8. The figure shows three water molecules (A, B, and C), which were placed in the cytoplasmic channel as reported by Humphrey et al. (1994). At the M₈ stage, two of the three waters (A and B) strongly hydrogen-bond with each other. Water A also hydrogen-bonds to the hydroxyl moiety of Asp-96. Water B also forms hydrogen bonds with the hydroxyl group of Thr-46 and the carbonyl group of Phe-219. Water C weakly connects with the hydroxyl moiety of Thr-46 and with water B, the angles of both connections not being optimal. Water C did not exhibit any further hydrogen bond, i.e., this water is not in an energetically favorable situation in M₈.

In the counterion region and in the extracellular channel, three water molecules (F, G, and H) arrange themselves to connect to the hydroxyl group of Tyr-57 and to the oxygen

![FIGURE 4](image)

Calculated carriers carried out for the L₁ pathway (a), and for the L₂ pathway (b). The notations used in the figure are: TP, transfer of a proton from the retinal Schiff base to Asp-85, followed by 200 steps of conjugate gradient minimization; Eqm, 20-ps molecular dynamics equilibration at T = 300 K, followed by a 200-step minimization; Mwat - movement of a water molecule to a position into the cytoplasmic channel, again followed by a 200-step minimization; SA, simulated annealing employing the soft constraints for the single bond dihedrals along retinal’s backbone carrying out the following steps: 1) 100-step minimization; 2) starting at 500 K: 100 fs simulation, use of T-coupling (Brünger, 1992) to rescale velocities with a friction constant of 100 ps⁻¹; then a 50-step minimization and reassignment of velocities corresponding to 490 K; again a 100-fs simulation and a 50-step minimization; same procedures at 480 K, 470 K, and so on, until 300 K was reached (the frequent reassignment of velocities facilitates the search for a wider conformational space, and the 50-step minimization before reassignment of velocities helps to avoid instabilities due to velocity redistributions); 3) the process in (2) above was repeated, but starting from 400 K. After procedures 1–3 were completed, soft constraints for the single bond torsions along retinal’s backbone were released and a 10-ps molecular dynamics equilibration at T = 300 K was carried out; a 200-step minimization completed the simulated annealing.
bonyl oxygen of Arg-175 (a typical hydrogen bond of \( \alpha \)-helix) broke, and these moieties separated from a distance of 2.26 Å to a distance of 4.5 Å. The carbonyl of Arg-175 developed, and then a hydrogen bond to the hydroxyl of Thr-178 and helix F started to bend. We suggest that this bend widens the cytoplasmic channel of bR.

A significant conformational change is also exhibited by the cytoplasmic portion of helix G at the \( M_s \) stage. Helix G moved by \( \sim 1 \) Å away from the waters between Asp-96 and the retinal Schiff base toward helix F. Our simulations appear to be in agreement with observations of changes in bR’s electron density at the \( M_{412} \) stage as observed in Hauss et al. (1994). In our simulations, helix E experienced a translational shift of about 2 Å toward the center of bR, but otherwise the conformation of helix E did not change as much as that of helices F and G. No significant change was observed for helices A, B, C, and D.

Concomitant with the bend of helix F, the ring of Tyr-185 on the extracellular side of this helix moved relative to bR568 by about 3.8 Å away from the Schiff base toward the extracellular side and toward retinal’s \( \beta \)-ionone ring. This significant motion, shown in Fig. 10, is because of a weakening of the interaction of Tyr-185 with the hydrogen bond network of waters F, G, I, H (Fig. 8) after the retinal → Asp-85 proton transfer. As a result of the motion of Tyr-185, the extracellular side of helix F including Pro-186, moved down and away from the center of the protein by \( \sim 3 \) Å, whereas the hydrogen bond pattern of the helix backbone did not change significantly, i.e., no tilt developed in the portion of the helix around Tyr-183 and Pro-186. Pro-186 remains in close contact with retinal’s \( \beta \)-ionone ring throughout the early pump cycle up to the \( M_{412} \) stage. This implies that the conformational changes around Tyr-185 and Pro-186 are induced by the ring of retinal, as well by the hydrogen bond between Tyr-185 and the water-side group complex in the counterion region. Helix F around Trp-182 moved very little because of the bulky size of tryptophan, whereas the torsion angle of \( C_\beta \)-N of the Trp-182 backbone varied from 70.66° in bR568 and 66.89° in \( L_1 \) to 97.15° in \( M_s \). Hence, Trp-182 can be considered a hinge for the motion of the helix.

From \( M_\beta \) to \( M_r \)
When we applied further annealing to \( M_s \), the waters in the counterion region and in the extracellular channel did not change their configuration, whereas the waters in the cytoplasmic channel altered their arrangement. The rearrangement arose because waters, at this stage, did not fill any more all-accessible voids in the cytoplasmic channel; and therefore the waters became too mobile to form a stabilized configuration.

The conformational change of helix F and helix G introduced cavities in the cytoplasmic channel that provide space for further water molecules (Fig. 10). Accordingly, we placed one water (water D), originally positioned on the far extracellular site in \( M_s \), between the retinal Schiff base and
water C. Water D did not remain in this position, but rather moved toward Asp-96 to connect with waters B and C. This placement established a connection between the hydroxyls of Asp-96 and of Thr-46 and the carbonyl of Lys-216 through waters A, B, C, and D.

In $M_c$, waters C and D remained at their place, but the hydrogen bond between water A and the hydroxyl of Asp-96 broke, and these waters moved toward the Schiff base. The four water molecules, A, B, C, and D, formed a hydrogen bond network.

In $M_d$, the four water molecules in the cytoplasmic channel kept the same arrangement as in $M_c$. One interesting feature is that waters B and D exchanged their position, which implies that there were still cavities in their vicinity. A small rearrangement between these four water molecules indicates a suboptimal hydrogen bond network in $M_d$ that may drive the subsequent dynamics of the $M_{412}$ intermediate.

At the $M_a$ stage, the hydrogen bonds between waters A, B, C, and D became even more unstable, the water closest to the retinal Schiff base, i.e., water D, still being too distant to form a hydrogen bond with retinal. However, during the equilibration of $M_a$, water D moved toward the Schiff base; the latter rotated to the cytoplasmic direction to finally establish a hydrogen bond with water D. This move lead to a breaking of the hydrogen bond between the Schiff base and water F, as shown in Fig. 8. The hydroxyl group of water F then hydrogen bonded with water D. Thus, the waters in the cytoplasmic channel and in the counterion region were connected with each other. At this stage water C hydrogen-bonded with the oxygen of the Thr-46 hydroxyl and with the carbonyl of Phe-219. Water A disconnected from the carbonyl of Phe-219 and moved toward the Schiff base together with water D.

In $M_e$, water D formed an even stronger hydrogen bond with the Schiff base, such that water F was driven away from the Schiff base, and induced a further rotation of the Schiff base directly toward Asp-96. The conformation at the extracellular channel did not change significantly from $M_b$ to $M_e$.

The $M_f$ state

Annealing of $M_e$ did not lead to significantly new conformations. Because enough space exists in the cytoplasmic channel for another water molecule, we moved water E from the far extracellular site to the place between the hydroxyl of Asp-96 and water C in an attempt to build a continuous hydrogen bond network between Asp-96 and
retinal. This placement resulted in structure $M_g$, which formed the desired hydrogen bond network between Asp-96, Thr-46, Phe-219, Lys-216 and the Schiff base, as shown in Fig. 11.

In the counterion region the hydrogen bonds between waters E, F, G, I, and H assumed an optimal geometry such that the arrangement of waters and residues around the Schiff base became more compact. An analysis of simulations also revealed that the water molecules in $M_g$ are less mobile than in the previous stages of the $M_{412}$ intermediate.

Recent FTIR measurements revealed that Tyr-185 undergoes a significant structural change during the $bR_{368} \rightarrow N_{520}$ transition (Ludlam et al., 1995). Our simulations show that the geometry of $M_g$ is close to that of $N_{520}$ formed by Asp-96 $\rightarrow$ retinal protonation around Tyr-185. The conformation of Tyr-185 in $M_g$, and consequently in $N_{520}$, is similar to that of $M_a$.
FIGURE 10 *Top:* View of the seven trans-membrane helices of bR<sub>568</sub> and M.<sub>a</sub> The tilt of helix F in M.<sub>a</sub> is seen to widen the cytoplasmic channel. *Bottom:* Conformation of helix F and its environment in bR<sub>568</sub> and M.<sub>a</sub>. Dashed lines between atoms represent possible hydrogen bonds.

i.e., it moves more than 3 Å away from the Schiff base compared with its position in the structure of bR<sub>568</sub>. Hence, our findings are in agreement with Ludlam et al. (1995).

**Summary**

Retinal's conformational dynamics at the M<sub>412</sub> stage are summarized in Figs. 5 and 12, which show the twist of the retinal Schiff base nitrogen from an orientation toward water F (which connects to Asp-85) to an orientation toward Asp-96. A comparison of Figs. 8 and 11 demonstrates how water molecules disconnect their hydrogen bond network from Asp-85 and establish a network between Asp-96 and retinal. Our simulations reveal clearly that the M<sub>412</sub> intermediate, in the case of the L<sub>1</sub> candidate of L<sub>550</sub>, realizes the protein switch function necessary for proton pumping. The tilts of helix F and retinal in simulations are in agreement with observations.

**Simulation of an M<sub>412</sub> intermediate starting from L<sub>2</sub>**

We will now consider the evolution of bR in the case that the M<sub>412</sub> stage is initiated through retinal → Asp-85 proton transfer of L<sub>2</sub>.

The M<sub>a</sub> state

Proton transfer from retinal to Asp-85 in L<sub>2</sub> and equilibration lead to a structure denoted as M<sub>a</sub>. Fig. 13 presents the corresponding geometry of retinal and compares it to the geometry of retinal in bR<sub>568</sub>. One notes that retinal in M<sub>a</sub> is shifted toward the cytoplasmic side, the methyl carbon C<sub>20</sub> experiencing a shift of 4.64 Å, which is much larger than that experienced at this stage in the L<sub>1</sub> pathway. In the present case, the Schiff base nitrogen points toward the extracellular side, i.e., away from the proton donor Asp-96. The changes seen are at variance with the observations reported in Heyn and Otto (1992), which showed that in the M<sub>412</sub> intermediate the C<sub>20</sub> methyl group shifts by only 1.7 Å toward the cytoplasmic side of the membrane relative to the bR<sub>568</sub> state.

Fig. 14 provides 2 views of retinal, of key amino acid side groups and of key water molecules. The three water molecules (A, B, C) in the cytoplasmic channel form a hydrogen bond network involving Thr-46 and the carbonyls of Phe-219 and Lys-216. These waters do not hydrogen-bond with Asp-96, nor, because of the opposite orientation, with retinal’s Schiff base. In the counterion region and in the extracellular channel, a hydrogen-bond network of waters is formed with three water molecules
The structure M\(_{1a}\) exhibits a total RMSD from the structure of L\(_2\) of 1.63 Å, which is significantly smaller than the RMSD experienced at this stage for the model of M\(_{412}\) based on the L\(_1\) state. Fig. 15 presents the overall structure of bR in M\(_{1a}\). Helices F and G show only small changes compared to bR\(_{568}\); in particular, a bent of helix F does not arise. Helices D and E exhibit larger RMSD than others.

**From M\(_{1b}\) to M\(_{1a}\)**

In M\(_{1b}\), the structure of the Schiff base and the configuration of the cytoplasmic channel is almost the same as in M\(_{1a}\), except that water B and Asp-96 approach each other to form a strong hydrogen bond. After the annealing of M\(_{1b}\), the Schiff base still pointed down. Only during the equilibration stage after the annealing did the Schiff base gradually turn toward Asp-96 through isomerization around the 14–15 bond of retinal. At the M\(_{1a}\) stage, the Schiff base pointed toward Asp-96 (see Fig. 13), but did not engage in a hydrogen bond with the waters in the cytoplasmic channel. Further annealing did not lead to formation of a hydrogen bond network to connect the retinal Schiff base to Asp-96. We conclude from this that at least one more water molecule needs to be added to the cytoplasmic channel to build the needed connection between retinal and Asp-96. Accordingly, we placed a water molecule between the retinal Schiff base and its closest water to form M\(_{1a}\). The overall structure of the protein did not change as a result. Water B and Asp-96 separated to assume a distance of 2.66 Å between the carboxyl hydrogen of Asp-96 and the oxygen of water B.

**The M\(_{1e}\) state**

After another round of simulated annealing we reached M\(_{1e}\), which is represented in Fig. 16. At this stage, one can...
retinal, the simulations indicate that the sequence $M_{Ia}$ to $M_{Ie}$ can also serve as a proton switch of bR. However, the $M_{Ia}$ to $M_{Ie}$ sequence does not involve a tilt of helix F (cf. Fig. 9 for the $M_{a}$ state) and implies a tilt of retinal that is at variance with observations.

**Interaction energies**

Important information on the role of the $M_{412}$ intermediate is also conveyed by the interaction energies between the retinal Schiff base or key amino acid side groups and water, as presented in Table 1, as well as by the energies for possible proton transfer transitions presented in Table 2. These energies do not provide quantitative measures of respective $pK_a$ values.

**DISCUSSION**

Our molecular dynamics simulations allow us to discuss several key aspects of the $M_{412}$ intermediate of bR, namely, the interactions that induce the $L_{550} \rightarrow M_{412}$ transition, the role of helix F during the $M_{412}$ intermediate, the role of the $M_{412}$ state as a proton switch, the forces driving the decay of the $M_{412}$ state toward the $N_{520}$ state, and the heterogeneity of the $M_{412}$ intermediate. We also briefly comment on the simulated annealing method used in our simulations.

**Driving forces for the $L_{550} \rightarrow M_{412}$ transition**

The $L_{550} \rightarrow M_{412}$ transition involves the transfer of the retinal Schiff base proton to Asp-85. A key question regarding this transition is which interactions stabilize the protonation of Asp-85. As pointed out by Hwang and Warshel (1988), an ion pair’s microenvironment plays the essential role in the ion’s stabilization. Our simulations indicate that major factors of this environment in bR are the bound waters (in this case, waters bridging the protonated Schiff base and negative charges in its vicinity), and their geometry.

In fact, our simulations show that in the $L_{550}$ intermediate, the water molecules that bridge the Schiff base and Asp-85 do not fit as tightly as in bR$_{568}$ (Humphrey et al., 1995). This is clearly evident from the entries of Table 1, which indicate that the interaction energies of water with the retinal Schiff base and with Asp-85 in the $L_{550}$ intermediate are less than those in bR$_{568}$. These observations support the suggestion based on model compounds and artificial pigments studies (Gat and Sheves, 1993; Roussou et al., 1995) that a major factor that controls the $pK_a$ of the ion pair in bR binding site involves specific geometrical arrangements of the donor and acceptor groups and particularly the bound waters between them that allow effective stabilization of the ion pair. Observations of bR mutants also revealed that the arrangement of bound water exerts a large degree of control over the proton transfer process in the $L_{550} \rightarrow M_{412}$ transition. Neither in the case of an Asp212Asn mutation (Stern
and Khorana, 1989; Otto et al., 1990; Rothschild et al., 1990) nor in the case of a Tyr57Asn mutation (Soppa et al., 1989) does the M_{412} intermediate form at pH = 7. Our simulations show that the mentioned residues play an important role in the hydrogen bond network. We suggest that mutation of Asp-212 or Tyr-57 alters the water environment around the Schiff base during the photocycle and thereby renders the L_{550} → M_{412} transition unfavorable. The importance of protein structural changes for the stabilization of M_{412}, i.e., for change of the pK_a value of the protonated Schiff base and a shift of the equilibrium toward the unprotonated Schiff base, was suggested previously (Koutalos et al., 1990). Our simulations suggest a specific structure for the hydrogen bond network of water between the Schiff

FIGURE 14 Structure of M_{412} in the vicinity of the Schiff base, seen from two different directions. Dashed lines between atoms represent possible hydrogen bonds.

FIGURE 15 Comparison of the overall structures of M_{412} and M_{412}. From left to right, the ribbons represent helices E, F, G and A and the thin lines represent helices D, C, and B.
base, Asp-212, Arg-82, Thr-89, and Tyr-57, which plays an important role in stabilizing the M$_{412}$ intermediate.

Following proton transfer to Asp-85, the interaction between Asp-85 and water is weakened significantly, as shown in the entries of Table 1 and as illustrated through the hydrogen bond network shown in Figs. 8 and 14. These results are consistent with FTIR measurements (Engelhard et al., 1985, Braiman et al., 1991) that indicate that Asp-85 is in a hydrophobic environment at the M$_{412}$ stage.

### TABLE 1 Interaction energies between key groups and water

<table>
<thead>
<tr>
<th>Stage</th>
<th>Schiff base (N or NH)</th>
<th>Asp-85 (COO$^-$ or COOH)</th>
<th>Asp-96 (COOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bR$_{558}$</td>
<td>−9.3</td>
<td>−22.5</td>
<td>−8.8</td>
</tr>
<tr>
<td>$L_1$</td>
<td>−6.6</td>
<td>−13.2</td>
<td>−7.4</td>
</tr>
<tr>
<td>$M_a$</td>
<td>−14.4</td>
<td>−0.8</td>
<td>−8.5</td>
</tr>
<tr>
<td>$M_b$</td>
<td>−14.3</td>
<td>−3.1</td>
<td>−9.7</td>
</tr>
<tr>
<td>$M_c$</td>
<td>−16.0</td>
<td>−2.2</td>
<td>−0.5</td>
</tr>
<tr>
<td>$M_d$</td>
<td>−15.8</td>
<td>−4.6</td>
<td>−1.6</td>
</tr>
<tr>
<td>$M_e$</td>
<td>−14.2</td>
<td>−1.8</td>
<td>−0.4</td>
</tr>
<tr>
<td>$M_f$</td>
<td>−10.2</td>
<td>−2.0</td>
<td>−0.3</td>
</tr>
<tr>
<td>$M_g$</td>
<td>−12.3</td>
<td>−3.9</td>
<td>−6.6</td>
</tr>
<tr>
<td>$L_2$</td>
<td>−1.8</td>
<td>−26.8</td>
<td>0.0</td>
</tr>
<tr>
<td>$M_{I_a}$</td>
<td>1.0</td>
<td>−8.1</td>
<td>−0.3</td>
</tr>
<tr>
<td>$M_{I_b}$</td>
<td>1.4</td>
<td>−1.7</td>
<td>−2.7</td>
</tr>
<tr>
<td>$M_{I_c}$</td>
<td>−1.3</td>
<td>−12.3</td>
<td>−7.1</td>
</tr>
<tr>
<td>$M_{I_d}$</td>
<td>−13.2</td>
<td>−11.1</td>
<td>−1.4</td>
</tr>
<tr>
<td>$M_{I_e}$</td>
<td>−14.5</td>
<td>−5.0</td>
<td>−5.7</td>
</tr>
</tbody>
</table>

The values (in units of kcal/mol) listed in the table are the sum of electrostatic and explicit hydrogen bonding interaction energies between the particular group listed in the table and all the water molecules placed in bR. Structures used for the calculation of these energies were obtained through energy minimization of the respective equilibrated structures.

As shown in Table 2, our simulations indicate that the L$_{550}$ → M$_{412}$ transition is a strongly activated process if we view $M_a$ or $M_{I_a}$ as a product of $L_1$ or $L_2$. The energy for an immediate proton transfer in the $L_1$ or the $L_2$ state is actually positive (52 or 60 kcal/mol respectively). The transition becomes possible only through stabilization of the resulting state by 74 kcal/mol in the subsequent 20-ps equilibration process between $L_1$ and $M_a$ involving an optimal hydrogen bond network in the counterion region (the energy difference before and after the proton transfer, i.e., between $L_1$ and $M_a$ is now −22 kcal/mol). In contrast, transfer of a proton in bR$_{568}$ from the Schiff base to Asp-85 is also strongly activated (62 kcal/mol), but in the latter case subsequent equilibration does not stabilize the system as strongly as in the $L_1$ case (37 kcal/mol). Although the numbers of energies may not be exact because of errors in

### TABLE 2 Energy difference for proton transfer reactions

<table>
<thead>
<tr>
<th>Transition</th>
<th>Transfer (a)</th>
<th>Transfer and eqn (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bR$_{568}$ (retinal → Asp-85)</td>
<td>62</td>
<td>37</td>
</tr>
<tr>
<td>$L_1$ (retinal → Asp-85)</td>
<td>52</td>
<td>−22</td>
</tr>
<tr>
<td>$L_2$ (retinal → Asp-85)</td>
<td>60</td>
<td>14</td>
</tr>
<tr>
<td>$M_a$ (Asp-96 → retinal)</td>
<td>−30</td>
<td>−72</td>
</tr>
<tr>
<td>$M_{I_a}$ (Asp-96 → retinal)</td>
<td>−11</td>
<td>−45</td>
</tr>
</tbody>
</table>

Energies (in units of kcal/mol) connected with various proton transfers, i.e., the differences in the total potential energy of bR before and after the respective transitions. The energies after the transition were calculated in two ways: (a) transfer of the proton from the Schiff base to Asp-85 to form a possible M$_{412}$ state, or transfer of the proton from Asp-96 to the Schiff base leading to a possible N$_{520}$ state; (b) same as (a), but including a 20-ps equilibration at 300 K after the proton transfer. Energies were calculated from minimized structures.
energy calculations, the strong tendency appears to show that the conformational rearrangement during proton transfer in the $L_{550}$ state is very important for the stabilization of the $M_{412}$ intermediate.

**Shift of retinal spectrum in $M_{412}$**

The formation of optimal hydrogen bonds around the retinal Schiff base linkage in the initial $M_{412}$ state can also explain why the spectrum of retinal is strongly red-shifted at this stage of the photocycle relative to the spectrum of the unprotonated Schiff base of retinal in solution, the latter absorbing at 360 nm (Gat and Sheves, 1994). It had been suggested previously that this red shift originates from an effective hydrogen bond of the Schiff base with internal water or with protein residues (Gat and Sheves, 1994). Our simulations suggest that the red shift is due to hydrogen bonding between a structured water and the Schiff base. As shown in Table 1, as well as indicated in the structures shown in Figs. 8 and 11, the Schiff base has significantly stronger interaction with water in all stages of $M_{412}$ in the $L_1$ pathway and in later stages of the $L_2$ pathway than it has in the $L_{550}$ intermediate or in $bR_{568}$.

**The role of helix F**

The bend of helix F is a key attribute of the $M_{412}$ intermediate which raises the question of what interactions induce it. Unfortunately, our simulations do not give a clear answer. To understand the mechanism of the bending of helix F we have carried out simulations with modified structures. The bend arises only after deprotonation of retinal in an $L_1$ conformation and does not arise after deprotonation of retinal with $bR$ in an $L_2$ conformation. FTIR measurements indicate that amide bonds experience structural alterations following $M_{412}$ formation (Braiman et al., 1987). The changes might be associated with the conformational changes of the F and G helices, characterized by the destruction of the hydrogen bonds along $a$-helices between amide groups and carbonyls, including changes of the hydrogen bonds of carbonyls of Lys-216 and Phe-219 on helix G. Recent studies have shown that the Lys-216 carbonyl exhibits a very low stretching frequency in the $M_{412}$ intermediate, and it has been suggested that the carbonyl experiences a strong hydrogen bond with bound water (Takei et al., 1994). This suggestion is supported by our simulations. The conformational changes of helical backbones are also in agreement with the finding reported in Subramanian et al. (1993), except that a translational change of helix E predicted by our simulations was not observed.

In our simulations, the bend of helix F diminishes in going from $M_s$ to $M_d$, and disappears altogether for $M_c$ when the waters in the cytoplasmic channel become ordered and form a hydrogen bond network with the Schiff base. This result suggests that the bend of helix F is affected by the waters in the cytoplasmic channel. Indeed, simulations in which the three water molecules in the cytoplasmic channel were removed at the $M_s$ stage yielded a significantly smaller bend of helix F. In the case that all 16 water molecules in the bR interior are removed at the $M_s$ stage, no significant bend of helix F arises. This finding might explain the previously reported electron diffraction data (Gläser et al., 1986), which did not reveal protein structural changes at the $M_{412}$ stage of bR’s photocycle for dried, glucose-embedded specimens. As discussed in Results, the waters in the cytoplasmic channel are destabilized at the $M_s$ stage. We suggest that because of the proton transfer from the protonated Schiff base to Asp-85, the dipole moment of the Schiff base and its counterion region changes, which affects the water in the cytoplasmic channel and ultimately helix F.

In order to investigate further the causes for the bending of helix F following retinal $\rightarrow$ Asp-85 proton transfer in $L_1$, we also carried out the simulations for a protein structure characterized by removal of the retinal chromophore from $L_1$ structure. A proton was transferred from Lys-216 amino group to Asp-85. In addition we tested retinal $\rightarrow$ Asp-85 proton transfer in $bR_{568}$. Neither of the above simulations induced significant bends of helix F after 20-ps dynamics at 300 K. The findings show that retinal contributes to the bend of the helix. Apparently, the restructuring of the water and amino acid side groups in the counterion region following proton transfer, exhibiting an energy of stabilization of 74 kcal/mol, also contributes to the bending of the helix.

**The $M_{412}$ state as a proton switch**

The $M_{412}$ state plays a key role as a proton switch in the pump cycle of bR, $M_{412}$ being formed by transfer of a proton that feeds to the extracellular space and $M_{412}$ decaying through transfer of a proton fed from the cytoplasmic side. Our simulations suggest specific roles of retinal, of the protein, and of the internal waters in this respect. As pointed out above, experimental evidence favors the simulated $M_{412}$ intermediate that results from the $L_1$ structure for $L_{556}$ suggested in Humphrey et al. (1995) rather than an $M_{412}$ derived from the $L_2$ structure. Fig. 12 summarizes the mechanism of the light-driven proton pump that emerges from this finding. The role of the phototransformation is to prepare a stereochemically specific intermediate, namely, the $L_1$ structure, in which the plane of the Schiff base nitrogen and its two neighboring carbon atoms points parallel to the membrane plane toward a water molecule that provides a direct hydrogen bond contact to the primary proton acceptor Asp-85. Immediately after the proton transfer, retinal remains in the stated geometry, but at the late stage of $M_{412}$ the retinal nitrogen turns toward Asp-96, i.e., toward the proton donor. The motion, triggered by cytoplasmic water approaching the Schiff base linkage, involves only a small redirection of retinal, which is in agreement with linear dichroism data (Hehn and Otto, 1992), but requires a significant motion of the chain of Lys-216 (Fig. 7) as shown in Gat et al. (1992).
Our simulations suggest that in the early stage of $M_{412}$ the dipole moment of the complex of water, retinal, and amino acid side groups in the cytoplasmic region is changed, which influences the arrangement of water in the cytoplasmic channel and triggers a conformational change of the protein on the cytoplasmic side, in particular, helices F and G. An opening of the cytoplasmic channel allows more water to enter the channel, to approach the Schiff base linkage region, and to form a complete hydrogen bridge network between Asp-96 and the reoriented retinal Schiff base nitrogen. Comparing the structure of $M_a$ and $M_6$ in Figs. 8 and 11, one can see that water molecules around the Schiff base in $M_6$ engage in more extended hydrogen bonds than in the $M_a$ stage. Such rearrangement of water implies a significant ordering of water and, hence, an entropy reduction as is, in fact, observed in Ort and Parson (1979) and Váró and Lanyi (1991a). The retinal, the protein, and the water in the late stage of $M_{412}$ are perfectly set up for the Asp-96 $\rightarrow$ retinal proton transfer connected with the decay of $M_{412}$ and the formation of the $N_{520}$ intermediate.

Decay of $M_{412}$

Experimental evidence indicated that the decay of $M_{412}$ is significantly slowed down under low humidity conditions (Korenstein and Hess, 1977, Lechner et al., 1994) or by using osmotically active solute (Cao et al., 1991), which shows that water plays an important role in the Schiff base protonation and in the subsequent stages associated with the decay of $M_{412}$. The waters in the cytoplasmic channel, which connect Asp-96 to the Schiff base, are located between helices B, C, and G. These waters make direct contact with the hydroxyl group of Thr-46 on helix B, the Lys-216 and Phe-219 carboxyls on helix G, constituting a hydrophilic environment. The mentioned groups stabilize the water chain between Asp-96 and the Schiff base through hydrogen bonds as born out by a Thr-46 to Val-46 mutation, which shows a slow $M_{412}$ decay (Martí et al., 1991; Subramaniam et al., 1991; Brown et al., 1993, 1994). The other side of the water chain is surrounded by hydrophobic groups, i.e., Phe-42 on helix B, Leu-93 on helix C, Phe-219 on helix G, Val-49 on helix B the methyl group of Thr-89 on helix C, and the C$_{20}$ methyl group of retinal. These groups interact unfavorably with waters and force them to develop good hydrogen bonds with each other and with the hydrophilic side of the cytoplasmic channel. As a result, the water may transfer a proton according to the Grothues mechanism (Brünger et al., 1983; Nagle and Tristram-Nagle, 1983). On the other hand, as pointed out by Warshel (1979), (1986), the hydrogen bond network between donor and receptor is not sufficient to make the proton transfer, and energetics is the final criterion to determine the possibility of proton transfer. A proton transfer chain requires a network of groups with low $pK_a$ (Warshel, 1978). The energetics of proton transfer in bR still needs further studies.

The simulations suggest that water reorganizations in the cytoplasmic region during $M_{412}$ is also made possible by conformational changes involving Leu-93, which allows easier access of water to the retinal’s Schiff base. This suggestion is corroborated by the observation that a Leu-93 to Ala-93 mutation exhibits faster $M_{412}$ decay than native bR (Subramaniam et al., 1991); the mutant provides more space in the channel to allow easier water access. The C$_{20}$ methyl group of retinal blocks Leu-93 from moving toward retinal and thereby maintains the structure of the water channel. It is predicted by the simulations that without the C$_{20}$ methyl group, Leu-93 will move toward the Schiff base and will block water from binding to the nitrogen of the Schiff base, such that a continuous proton transfer chain between Asp-96 and retinal is not established.

Heterogeneity of $M_{412}$

The simulations indicate that $M_{412}$ is a very heterogeneous intermediate and should be considered a sequence of states, i.e., a process, rather than a distinct state. Experiments showed that there are at least two components to the $M_{412}$ intermediate (Váró et al., 1992; Druckman et al., 1992; Takei and Lewis, 1993), a third component being suggested as well (Sasaki et al., 1992). A low temperature study demonstrated recently that $M_{412}$ consists of five substates (Friedman et al., 1994). The many $M_{412}$ substates characterized in our simulations fall into two classes, i.e., those before the turn of the retinal nitrogen from water F toward Asp-96 (Fig. 8) and those after the turn (Fig. 11).

The many metastable substates of $M_{412}$ captured in our simulations do not necessarily have to occur in the same sequence as in our simulation; the actual $M_{412}$ state, e.g., as observed spectroscopically, may constitute an ensemble of all these substates in quasi-equilibrium such that these $M_{412}$ substates may not be easily distinguished through their absorption spectra. The heterogeneity of the $M_{412}$ intermediate can also be understood through the suggestion in Austin et al. (1975) and Frauenfelder et al. (1979), i.e., that proteins exhibit a large number of nearly isoenergetic substates: the $M_{412}$ intermediate of bR appears to involve two classes of substates, $M_1$ and $M_2$, each class constituting a large number of metastable states.

Justification for simulated annealing

Finally, we would like to comment on the relationship between the actual dynamics of a protein and its description through simulated annealing. It is surprising that simulated annealing lead us to results in agreement with our observations. One possible reason is that the native bR is robust under changes of environmental factors like pressure and temperature, such that the key features of the $M_{412}$ intermediate are still preserved in a simulated annealing description. The results suggest to employ simulated annealing to other biomolecules to bridge the long time scale that current molecular dynamics simulations cannot access.
We thank J. Lanyi, B. Roux, J. Herzfeld, W. Humphrey, I. Logunov, and F. Zhou for helpful discussions. Most simulations presented in this paper were carried out on Silicon Graphics and Hewlett-Packard workstations operated by the Resource for Concurrent Biological Computing at the University of Illinois and were funded by the National Institutes of Health (grant PHS 5 P41 RR05969–04). Some simulations were done on a Cray 2 computer operated by the National Center for Supercomputing Applications at the University of Illinois and were funded by the National Science Foundation.

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