The phototropins are a family of blue light receptors that function as the primary blue light receptors regulating phototropism, chloroplast movements, stomatal opening, and leaf expansion in plants. Phot1, a member of this family, contains two FMN-binding domains, LOV1 and LOV2, within the N-terminal region and a C-terminal serine-threonine protein kinase domain. Light irradiation of oat phot1 LOV2 produces a cysteinyl adduct (Cys-39) at the flavin C(4a) position, which decays thermally back to the dark state. We measured pH and isotope effects on the photocycle. Between pH 3.7 and 9.5, adduct formation showed minimal pH dependence, and adduct decay showed only slight pH dependence, indicating that the pK values of mechanistically relevant groups are outside this range. LOV2 showed a nearly 5-fold slowing of adduct formation in D2O relative to H2O, indicating that the rate-limiting step involves proton transfer(s). Light-induced changes in the far UV CD spectrum of LOV2 revealed putative protein structural perturbations. The light minus dark CD difference spectrum resembles an inverted α-helix spectrum, suggesting that α-helicity is reversibly lost upon light irradiation. Decay kinetics for CD spectral changes in the far UV region occur at the same rate as those in the visible region, indicating synchronous relaxation of protein and chromophore structures.

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centrifuged and/or filtered through a 0.2-μm filter prior to use. Protein concentrations were determined by absorption spectroscopy using a Hewlett Packard HP8452 diode array spectrophotometer with ε447(LOV2) = 13,800 M⁻¹ cm⁻¹ (14).

Light-induced Absorption Changes at Short Times—Difference spectra in the 30-ns to 690-ms time window were collected on an instrument described previously (21). A dye laser pumped by the third harmonic of an Nd:YAG laser provided a 10-ns, 80-μm/s² light pulse at 477 nm. A fresh sample was provided for each laser flash, allowing the averaging of absorbance data of several samples. The laser pulse traversed the sample perpendicular to the path of white light from a flashlamp that was used to probe the absorbance change in the sample. The optical path lengths for the probe light and laser were 2 and 0.5 mm, respectively. The white light used to probe absorbance was linearly polarized at the magic angle (54.7°) relative to the laser polarization axis to prevent rotational diffusion artifacts (22). The temperature for all measurements was about 20 °C.

Light-induced Changes at Long Times—As described previously (15), difference spectra in the 1–100-s time range were collected on an HP8452A diode array spectrophotometer at room temperature. The optical path length was 1 cm, and the excitation pulse was provided by a white light camera strobe flash.

Global Analysis—Data were analyzed using programs written in a Matlab environment (The Mathworks, Natick, MA) as described previously (15). Briefly, data matrices were constructed and subjected to singular value decomposition followed by global exponential fitting (23, 24). Kinetic changes at all measured wavelengths are decomposed into a sum of exponential components. The exponents contain the apparent rate constants for the observed kinetic changes, and the amplitudes at different wavelengths represent the spectral changes associated with the exponential process and are called the b-spectra (23, 25).

D2O Exchange—Protein samples were divided into two equivalent aliquots and were lyophilized overnight in the dark as described previously (15). One aliquot was then reconstituted in H₂O and the other in D₂O.

pH Titration—Protein samples (5 mM Tris, 10 mM NaCl) began at pH 8. The pH was changed in a stepwise manner using 0.5–1 mL HCl or NaOH and was monitored with a Corning Digital 110 meter and a Beckman Futura (model 511063; Fullerton, CA) semimicro AgCl combination electrode.

CD Spectroscopy—Protein CD spectra were recorded using an Aviv 60DS CD spectrometer. A lid for the CD instrument was constructed containing shutters for blocking the CD lamp and detector, and a hole was made for a light guide that fit directly into the top of the cuvettes. Thus, samples placed into the CD sample chamber were in complete darkness while the CD lamp and detector were blocked and the external lamp was off. The adduct form was induced by 20 s of white light irradiation from an external 100-Watt tungsten halogen lamp via the light guide. We observed no spectral or kinetic differences resulting from the use of white or blue light. The CD measuring lamp itself was not observed to be actinic. Full spectra were recorded at 2 °C in order to prolong the adduct form (14). Spectra were smoothed using the smoothing program in SigmaPlot (SPSS Science). Using the same irradiation method described above, we monitored relaxation kinetics by CD at wavelengths of 450, 290, 222, and 208 nm at 20 °C. Control experiments were conducted at 500 and 250 nm, wavelengths at which little difference is observed between dark and light spectra (data not shown). Points were recorded every 1 s for 700 s at 20 °C. In the visible/near UV region (260–500 nm) 15 μM protein was used in a 1-cm path length rectangular cuvette; in the far UV region (190–250 nm), 7 μM protein was used in a 0.1-cm path length rectangular cuvette. Relaxation kinetics by CD were also measured at 10 and 4 °C for calculation of activation energy.

RESULTS

FMN-cysteinyl Adduct Formation from the FMN Triplet State Is Nearly 5 Times Slower in D₂O than in H₂O—We previously demonstrated by nanosecond laser spectroscopy (see “Materials and Methods” and Ref. 15) that light irradiation of LOV2 produces the FMN triplet state, characterized by a broad absorption band at about 660 nm (LOV2, λ₆₆₀) and, subsequently, the flavin-cysteinyl adduct with characteristic absorption at 390 nm (LOV₂⁺₃₉₀) (15). Using the same technique, we measured changes in the absorption difference spectrum of LOV2 in H₂O and in D₂O between 30 ns and 690 ms after an excitation flash. The flavin triplet state decayed in H₂O with an apparent time constant² of 3 μs (data not shown), slightly slower than our previously reported value (15). A small difference in temperature could account for the difference in rate. Fig. 1A shows the absorption difference spectra of LOV2 in D₂O. Global exponential analysis of the measured difference spectra indicates a single first order process with an apparent time constant of 14 μs, almost 5 times slower than in H₂O. The residuals (the difference between spectral data and calculated data from the exponential fitting) show no significant spectral features. The inset of Fig. 1A shows the relative first-order rates in H₂O and D₂O, approximated by the decay of the 660-nm band alone.

Fig. 1B shows the corresponding b-spectra that resulted from the global exponential analysis. The single exponential fit indicates that two unique spectra (represented by b₁ and b₂) exist over the time course of the measurements, where each spectrum represents at least one species. The b₁-spectrum represents the decay difference between the spectra of the first (present at 30 ns) and final (persisting at 690 ms) intermediates and corresponds to the apparent decay time constant of 14 μs. The b₂-spectrum is the spectrum of the final intermediate referenced against the ground form. We showed previously that the b₁-spectrum resembles the difference spectrum of the FMN triplet state and that the b₂-spectrum is spectrally consistent with the ground state.

In this work, the time constant (denoted τ) is used interchangeably with lifetime, which refers to 63% (e.g. (1/e) reaction completion and is equal to the reciprocal of the apparent rate constant, k.app. Accordingly, for branched reactions, k.app = 1/τ.app = 1/τ₁ + 1/τ₂ + . . . + 1/τn and τ.app = (1/k.app) (ln 2).

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with a flavin-cysteinyl adduct (15). The presence of an isosbestic point (at about 420 nm) is also consistent with a two-state system, as observed previously (15). Therefore, the $b_1$- and $b_0$-spectra in Fig. 1B, which are nearly identical to those produced in H$_2$O (data not shown) (15), represent the same transitions of the triplet state to the FMN-cysteinyl adduct and the ground form, respectively, as reported earlier (15).

Absolute spectra for the triplet state (LOV2$^{280}$-660) and adduct form (LOV2$^{260}$-390) were calculated previously (15) by adding to each intermediate spectrum (calculated from the $b$-spectra) enough ground state absorption spectrum to remove the bleach at 450 nm fully. Twice as much ground state absorption spectrum was required to remove the bleach in the intermediate spectrum (calculated from $b_1$) as in the second intermediate spectrum (calculated from $b_0$). Hence, twice as much bleach at 450 nm exists in the triplet state as in the adduct form, and therefore the triplet state apparently decays into equal amounts of ground state and adduct. The apparent time constant of 3 $\mu$s for triplet decay in H$_2$O therefore represents two equivalent time constants$^2$ of 6 $\mu$s (for return to ground state and for adduct formation), as observed previously (15). In D$_2$O, the triplet state was also observed to decay equally to the adduct and to the ground state, resulting in time constants of 28 $\mu$s for both adduct formation and triplet relaxation to the ground state.

We showed previously that light irradiation of the LOV2C39A mutant, which lacks the sulfur group required to form the adduct, produces the triplet state, which then relaxes back to the ground state (15). In contrast to the wild type, only a negligible D$_2$O effect was observed for the truncated photocycle of the LOV2C39A mutant. The collected difference spectra are shown in Fig. 2A. The time constant observed for decay of the triplet state to the ground state is 72 $\mu$s in H$_2$O and 80 $\mu$s in D$_2$O; the relative rates are illustrated in the inset of Fig. 2A. As can be seen from the difference spectra, the decay of the 660-nm band is concomitant with the full recovery of the bleaching at 450 nm, indicating that no spectrally observable intermediates are formed during triplet decay. As shown in Fig. 2B, global analysis results in a $b_1$-spectrum that is consistent with the one from wild type LOV2 and a $b_0$-spectrum of zero.

Kinetics of FMN-cysteinyl Adduct Formation in H$_2$O Are pH-insensitive between pH 6.3 and 9.5—To probe the dependence of light-induced adduct formation on proton transfers further, we monitored triplet decay at the same time values used above from pH 6.3 to 9.5. We stayed within this range to minimize pH-dependent aggregation of LOV2 that occurs in solution at protein concentrations sufficient to show significant changes by absorption spectroscopy. The difference spectra and $b$-spectra obtained were the same as previously observed (data not shown). As shown in Fig. 3A, only a slight effect on the rate of adduct formation was observed from pH 6.3 to pH 9.5. The time constant for triplet decay is near 2.5 $\mu$s at all pH values measured. The relative pH insensitivity of adduct formation indicates that titratable groups in the chromophore and mechanistically relevant protein residues are not titrated within this pH range. This finding is consistent with our previous results that showed no change in chromophore fluorescence intensity between pH 5.5 and 10 (15).

Kinetics of FMN-cysteinyl Adduct Decay Show Minor Change between pH 3.7 and 9.8—We showed previously by absorption difference spectroscopy that the FMN-cysteinyl adduct decays back to the ground form with a half-life$^2$ of about 45 s (15). Here, we measured the rate of decay of the adduct form between pH 3.7 and 9.8. Slow chromophore release occurred below pH 5, as previously reported (15). However, the back-reaction kinetics were sufficiently slow to allow for longer signal integration times than were possible when measuring adduct formation. Further, samples restored to neutral pH showed recovered spectral and kinetic properties except for the expected loss of bleaching intensity due to some irreversible chromophore release. We were therefore able to acquire difference spectra down to pH 3.7.

The difference spectra were unaffected by the presence of released FMN, because it does not undergo a photocycle on the slow time scale of the protein. As shown in Fig. 3B, the rate of adduct decay was constant from high pH to about pH 7, below which a reproducible decrease in rate of about only 40% was apparent.

Circular Dichroism Spectrum of Phot1 LOV2 in the 190–500-nm Range: Dark and Light-irradiated States—The CD spectrum of the LOV2 ground form in the visible/near UV range was previously reported (14). We extended the measurement to the far UV range, which contains protein secondary structural information. The solid lines in Fig. 4 represent the CD spectra obtained for the ground state of LOV2 from 500 to 260 nm (Fig. 4A) and from 250 to 190 nm (Fig. 4B). As previously shown (14), there are negative CD bands in the 350–500-nm range that coincide in wavelength with all of the absorption bands of the chromophore. The visible wavelength bands represent only the FMN chromophore, because there are no protein groups absorbing at these wavelengths. In addition to these optically active chromophore bands, there are CD-active positive and negative bands in the protein aromatic residue region ($\lambda$$_{max}$ $\sim$280 nm), where flavin chromophores have substantially stronger absorption ($\lambda$$_{max}$ $\sim$270 nm) than individual aromatic protein residues.
In the far UV range (Fig. 4B), LOV2 shows a CD spectrum typical of a protein that contains significant fractions of α-helical and β-sheet structure (26). The peptide bond has strong absorption bands at about 215 nm and below 200 nm. CD bands in this region arise from electronic transitions of the peptide bond in particular structural conformations. The observed double minimum at 222 and 208 nm and maximum around 195 nm are signature features of helical secondary structure. These bands represent the $n \rightarrow \pi^*$ transition (222 nm) and exciton split $\pi \rightarrow \pi^*$ transition (190 and 208 nm) of peptide bonds and have high extinction coefficients in the α-helical conformation. For the β-sheet conformation, the transitions occur at 215 nm and at 175 and 198 nm, respectively, with lower extinction coefficients than observed for the α-helix conformation.

The primary absorption of the exciton split $\pi \rightarrow \pi^*$ transition occurs around 190 nm, such that its intensity is typically stronger than that at 208 nm. This ratio is not observed in the absolute far UV spectrum of LOV2, suggesting that there are contributions to the spectrum other than α-helix and β-sheet. The LOV2 fusion protein used in this work, shown in Fig. 5A, contains a 46-residue segment from the cloning expression vector and calmodulin-binding peptide, plus 10 native oat phot1 residues, that are N-terminal to the 107-residue LOV2 domain (11) and 50 additional native oat phot1 residues C-terminal to the LOV2 domain. The peptide bonds of these additional residues contribute to the far UV CD spectrum. The FMN chromophore is also expected to contribute to the spectrum, although only weakly. Free FMN in aqueous and organic solvents shows a positive CD band ($\lambda_{max} = 222$ nm) that is similar in magnitude to a single peptide bond (this work, data not shown) (27, 28). For comparison, Fig. 4B shows the molar ellipticity (CD magnitude) normalized to the concentration of protein (left axis) and peptide bonds (right axis). The LOV2 domain binds a single FMN chromophore such that the protein concentration is the same as the FMN concentration, whereas the peptide bond concentration is about 200 times larger because there is about one peptide bond per amino acid residue. The right axis in Fig. 4B, which represents the per residue CD intensity, is therefore about 200 times smaller in magnitude than that of the left axis. In order for FMN to make a significant contribution to the LOV2 far UV spectrum, the protein-bound FMN would have to acquire a CD magnitude large enough to compete with about 200 amino acid residues; in other words, the FMN would have to increase its CD magnitude by a few orders of magnitude.

The CD spectrum for LOV2 in the far UV range (Fig. 4B) is therefore expected to reflect primarily CD from the peptide bond and thus reflects protein secondary structure. The spectrum is consistent with the $\alpha + \beta$ tertiary structure classification (26), which is consistent with PAS domain structures in general and specifically with the predicted structure of oat phot1 LOV2 (14) and the crystal structure of phy3 LOV2 from the fern Adiantum capillus-veneris (29).

The dashed lines in Fig. 4 correspond to light-irradiated CD spectra (i.e. the CD spectra of the FMN-cysteinyl adduct) in the visible and UV ranges. The visible/near UV spectrum shows spectral changes consistent with the disappearance of a 450-nm species and the appearance of a 390-nm species. These changes are consistent with those reported previously (14). In the near UV (aromatic) region, a large, positive band appears at about 290 nm, also consistent with the previous report. Aromatic protein residues typically contribute to CD in this
Conserved residues are shaded. Upon light irradiation of LOV2 would correspond to a maximum loss of 10–15% of helicity. We fit the far UV CD difference spectrum to a linear combination of CD reference spectra (26) of various types of secondary structure. The fits were not unique (did not favor a particular combination of formed secondary structure); however, the loss of 10–15% helicity was necessary in all fits. The crystal structure (29) indicates that the LOV2 domain contains ~25% α-helical structure, so the overall change observed by CD is 10–15% of this (i.e. about 3% of the LOV2 domain secondary structure). However, the fusion protein used in this work has almost twice the molar mass of the LOV2 domain; therefore, the additional protein segments is unknown. Therefore, we cannot quantitate the fraction of lost helicity for the total fusion protein.

Protein and Chromophore CD Bands of Light-irradiated LOV2 Relax Synchronously in the Dark—We also used CD spectroscopy to determine whether protein structural perturbations could be distinguished kinetically from chromophore activity during the photocycle. As discussed above, changes in the visible and near UV regions of the CD spectrum reflect primarily chromophore activity, whereas changes in the far UV region probably reflect primarily protein secondary structure. From these spectra, we selected two wavelengths showing considerable changes upon light-irradiation from each region, namely 450, 390, 222, and 208 nm. We then monitored dark relaxation of the irradiated state of LOV2 by CD spectroscopy to see whether the expected protein bands (208 and 222 nm) show different rates from the chromophore bands (390 and 450 nm). As shown in Fig. 6, relaxation monitored at each of the four key wavelengths showed the same rate, with a half-life of about 45 s, consistent with absorption measurements. Relaxation was also monitored at these wavelengths in D2O, resulting in a slowing of the rate by a factor of about 3 (Fig. 7), consistent with our previous absorption measurement (15). No changes in CD were observed in control experiments in which dark relaxation was monitored at 250 and 500 nm, where no light-induced absorption changes occur in LOV2 (data not shown). Finally, the decay profile at 222 nm was also measured at 10 and 4 °C and was found to fit a single exponential with a rate of decay about 3 and 9 times slower than at room temperature (data not shown), respectively.

**DISCUSSION**

**D/H Exchange Demonstrates That the Rate-limiting Steps in Both FMN-cysteinyl Adduct Formation and Decay Involve Proton Transfers(s)—**Hydrogen transfer reactions require the breakage of a bond between hydrogen and the donor atom and the formation of a new bond between hydrogen and the acceptor atom. Deuterium transfer reactions are generally slower due to the larger mass of deuterium relative to hydrogen. Such kinetic slowing, referred to as a primary deuterium isotope effect, leads to a relatively large effect on the observed reaction.
LOV2C39A shows a minimal D2O effect. The LOV2C39A mutant lacks the sulfur group necessary to form the adduct and structural solvent effects, also contribute to observed solvent kinetic isotope effects (30). Such effects are often quite small in magnitude compared with primary effects. However, the effects are multiplicative, and therefore a large number of secondary effects can cumulatively contribute considerably to the observed kinetic isotope effect.

The results presented show that FMN-cysteiny1 adduct formation in phot1 LOV2 shows a large (5-fold) solvent D/H exchange kinetic effect, suggesting that formation or breakup of bonds involving hydrogen atoms are rate-limiting steps in this reaction. The crystal structure of phy3 LOV2 (19) indicates that multiple hydrogen bond perturbations occur in response to light, possibly constituting secondary isotope effects that are reflected in the observed 5-fold decrease in rate. No significant D2O-induced structural changes were observed by absorption and CD spectroscopies in the visible and UV ranges (data not shown), indicating that no secondary effects arise from this source.

The data presented here show that the rate of triplet decay in LOV2C39A shows a minimal D2O effect. The LOV2C39A mutant lacks the sulfur group necessary to form the adduct and therefore decays directly from the triplet state to the ground form (15). The observed D2O independence therefore indicates that triplet decay to the ground state does not involve rate-limiting proton transfers in the absence of the sulfur group.

The data presented also show that the triplet state in wild type LOV2 decays equally to the ground form and to the adduct form in D2O, although the respective rates are slowed about 5-fold relative to H2O. The preservation of the 50/50 split is consistent with our previous observation that the relative quantum yields of adduct formation are the same in H2O and in D2O (15). These data indicate that the processes of triplet decay to the ground form and adduct formation share a common rate-limiting step involving proton transfer(s). It is possible that a single molecular event, such as thiolate attack at C(4a) triggered by N-5 protonation, results in either adduct formation or intersystem crossing to the ground form. Such a mechanism is consistent with the observed 50/50 split between triplet decay to the ground form and adduct formation in both H2O and D2O. In the absence of this putative molecular event, such as when the sulfur group is not present, adduct formation would be precluded and triplet decay to the ground form would proceed by other (slower) processes. The observation that triplet decay in the LOV2C39A mutant is slow (time constant of 80 μs) is also consistent with a mechanism involving such a single molecular event.

The rate of decay of the adduct form measured by CD spectroscopy in the chromophore and protein spectral regions was found to be 3 times slower in D2O (data not shown) than in H2O. Further, this 3-fold rate effect is identical to that observed by light-induced absorbance (15) changes of the chromophore. These findings indicate that protein and chromophore relaxation processes are governed by common, rate-limiting proton transfer reaction(s). In addition, from the temperature dependence of the rate constants in H2O, we calculated that the activation energy barrier for the proton transfer rate-limiting step of adduct decay is about 55 kJ/mol, assuming Arrhenius behavior. This activation energy is within the energy range of a few hydrogen bonds.

The rate, especially in the case of rate-limiting proton transfer(s) (30–32).

Secondary isotope effects, which include hydrogen-bonding and structural solvent effects, also contribute to observed solvent kinetic isotope effects (30). Such effects are often quite small in magnitude compared with primary effects. However, the effects are multiplicative, and therefore a large number of secondary effects can cumulatively contribute considerably to the observed kinetic isotope effect.

FIG. 6. Dark relaxation of the LOV2 adduct monitored by circular dichroism at visible, near UV, and far UV wavelengths. Symbols represent raw data, and lines are first-order exponential fits that gave relaxation half-lives of 46 s at 450 nm, 47 s at 290 and 222 nm, and 51 s at 208 nm. These values are consistent with the half-life measured from absorption difference spectroscopy (45 s), which monitors only chromophore activity.

The Minimal pH Sensitivity for FMN-cysteiny1 Adduct Formation and Decay Suggests That Important Groups in the Rate-limiting Steps Have pK Values outside the Measured Range—Within the pH range studied, we observed neither significant changes in the absorption spectrum of the flavin chromophore in LOV2 nor substantial changes in the kinetics of adduct formation or adduct decay. Titration of residues that are involved in mechanistically relevant proton transfer(s) would be expected to show a relatively large rate effect, which was not observed for adduct formation or decay in the pH range studied. A broader pH range could not be tested due to slow release of the chromophore and/or protein precipitation that occurs at high and low pH.

The apparent pH independence of adduct formation between pH 5.5 and pH 9.5 was not unexpected, because most titratable protein groups including glutamate, aspartate, lysine, tyrosine, and arginine, have normal pK values outside this range. We have shown evidence that Cys-39 of LOV2 has an atypical pK of less than 4 (15). In addition to cysteine, the only other relevant group that shows apparent pK values in proteins in the range 5.5–9.5 is histidine (pK -6). The absence of significant pH effects on adduct formation indicates that either these groups do not interact with the chromophore and their ionization changes are inconsequential, or they also exhibit in LOV2 unusual pK values outside our experimental range. An addi-
tion.

We considered the possibility that the light-induced far UV CD changes observed for LOV2 are exclusively contributed to by the chromophore. Although free FMN shows extremely minor CD activity in far UV range, as discussed above, it is conceivable that FMN in the adduct form acquires absorption and strong CD activity in this wavelength region. In this case, the difference spectrum shown in the inset of Fig. 4B would represent the change in CD of the chromophore. However, as discussed above, it is unlikely that FMN could acquire the extremely large magnitude (at least a 100-fold increase) that would be needed to compete with the CD activity of the ~200 amino acid residues. Further, the double minimum present in both the far UV CD spectrum and the far UV CD difference spectrum is strongly indicative of helical structure and of mixed helix/sheet structure. A CD profile from FMN in the adduct form possessing coincidentally the identical electronic transitions with similar relative proportions is unlikely. Finally, light-induced protein structural perturbations in oat phot1 LOV2 are indicated by NMR (18) and by infrared (20).

We also considered the possibility that the far UV CD difference spectrum includes contributions from N- and C-terminal residues outside the LOV2 domain. As discussed above, the LOV2 fusion protein used in this work contains an expression vector segment and calmodulin-binding peptide at the N terminus of the LOV2 domain plus an additional 59 native phot1 residues (Fig. 5A). Protein secondary structural changes revealed by CD spectroscopy in the far UV range could include structural perturbations of these regions as well as of the LOV2 domain itself. If this were the case, remarkable signal transduction capability (from chromophore to extraneous protein segments) would be indicated. Indeed, fusion proteins containing both LOV1 and LOV2 domains from Arabidopsis (35), with LOV1 mutated to C39A, did show kinetic differences from the LOV2 fusion protein used here, suggesting that extension of the N-terminal region of LOV2 may impact light-induced protein behavior. We therefore interpret the observed light-induced far UV CD changes to reflect changes in protein secondary structure. These protein structural changes may be part of the signal transduction mechanism for oat phot1 LOV2.

Acknowledgments—We are grateful to Dr. John Christie for critical reading of the manuscript and for assistance with Fig. 5. We also thank Dr. David Kliger for generously allowing use of the spectroscopy facilities.

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J. Biol. Chem. 2003, 278:724-731.
doi: 10.1074/jbc.M209119200 originally published online October 30, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209119200

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