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Complexity of Bovine Rhodopsin Activation Revealed at Low Temperature and Alkaline pH

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ABSTRACT: The late intermediates involved in the activation mechanism of bovine rhodopsin are investigated by time-resolved optical absorption spectroscopy. Measurements from 10 μs to 200 ms after photolysis were carried out on membrane suspensions of bovine rhodopsin at a temperature of 15 °C and at pH of 7.3, 8.0, and 8.7. The time-resolved absorption spectra in the 330−650 nm range were analyzed by global exponential and kinetic scheme fitting methods. The results indicate an activation mechanism that is more complex than suggested previously. It involves interconnected branched pathways with two metarhodopsin I480 and two metarhodopsin II intermediates. The intermediates involved in this more complex mechanism need to be considered in spectroscopic studies that vary sample temperature and pH in order to enhance the presence of specific rhodopsin intermediates.

Rhodopsin is a light-sensitive G-protein coupled receptor (GPCR), found in the disk membranes of rod outer segment photoreceptor cells, which is involved in dim light vision in vertebrates. It has an 11-cis retinal chromophore, which is covalently bound to lysine-296 in the protein through a protonated Schiff base linkage. Upon absorption of light, the 11-cis retinal photoisomerizes to an all-trans retinal, triggering a series of chromophore and protein changes that lead to a transformation from an inactive state to an active state of the protein.1−3 The activated state then interacts with transducin, leading to an enzyme reaction cascade which results in blocking of ion pores in the rod outer segments. This causes hyperpolarization of the cells, which produces an electrical signal reporting on the detection of the light.4

The importance of this process for understanding visual transduction, as well as the function of GPCRs generally, has led to many investigations of the mechanism of rhodopsin activation. Early studies of the rhodopsin activation came from the pioneering work of George Wald and co-workers, using low-temperature trapping methods to stabilize intermediates involved in the activation process. Photoexcitation of bovine rhodopsin at 77 K revealed an intermediate whose spectrum is red-shifted relative to that of rhodopsin itself. Additional intermediates were found as the temperature was raised. The activation mechanism suggested by this technique is shown in Scheme 1.5,6

With the advent of fast laser excitation sources, it became possible to probe intermediates detectable at temperatures that are physiologically more relevant. Initial studies involved mapping out the early intermediates formed from nanoseconds to microseconds following excitation. The first intermediate, bathorhodopsin (Batho), emerges within a picosecond7−9 and then enters into equilibrium with a blue-shifted intermediate (BSI) during relaxation of the twisted chromophore in about 50 ns.10−12 In a few hundred nanoseconds, this equilibrium then decays into an intermediate whose spectrum is slightly blue-shifted from that of rhodopsin, the lumirhodopsin (Lumi) intermediate shown in Scheme 1.5 Subsequent studies of spectral changes which occur from microseconds to tens of milliseconds showed a complex branched scheme of interconverting intermediates (Scheme 2).13−18 Two Lumi forms, Lumi I and Lumi II, whose spectra are slightly shifted from one another, were later found and shown

Scheme 1. Activation Mechanism Suggested by Low Temperature Trapping Methods

Scheme 2. Activation Mechanism with the Branched-Square Scheme
to form an equilibrium mixture. The equilibrium mixture then branches to form two different intermediates, a Meta I intermediate absorbing at 480 nm, as shown in Scheme 1, and a Meta I-like form (occurring on a time scale similar to that of the “classical” Meta I) with a deprotonated Schiff base absorbing at 380 nm. To distinguish between the two Meta I intermediates, we denote them Meta I_{480} and Meta I_{380}. Then Meta I_{480} converts irreversibly into metarhodopsin II (Meta II), whose Schiff base is also deprotonated, while Meta I_{380} converts reversibly into Meta II as in Scheme 2. There have been a number of recent structural studies of the late photolysis intermediates, which show relaxation of the chromophore and the movement of the transmembrane helices as well as rearrangements of hydrogen-bonds. A number of studies also suggest that the active form of Meta II is formed with the movement of helix number six. As a result of this helix movement, a pH dependent final protonation of Glu-134 leads to Meta II−H⁺, which can bind the G-protein transducin and is thus the activated state.

Which branch of Scheme 2 dominates depends on the protein environment, such as pH, temperature, ionic conditions, and the presence of lipid membrane. In lipid membranes, lower temperature and higher pH favor Meta I_{480} formation from the Lumi II intermediate. Thus, low temperatures used in early trapping studies favor the lower branch, a mechanism similar to that of Scheme 1, while higher temperatures found under physiological conditions favor the upper branch of Scheme 2, formation of Meta I_{380} from Lumi II, as does protein solubilized in dodecyl-maltoside detergent. Early studies of the activation mechanism of human rhodopsin in our laboratory suggested that human and bovine rhodopsin follow similar mechanistic schemes with small differences in the kinetics and equilibria of the intermediates between the two species. Recently, having the more detailed mechanism shown in Scheme 2 available, comparative studies of bovine and human rhodopsin activation kinetics were revisited. The preliminary results on human rhodopsin indicate differences in the pH and temperature dependences of kinetics between the two proteins and show inconsistencies with the branched-square scheme (Scheme 2) at lower temperature and alkaline pH. This motivated us to re-examine the bovine mechanism under these conditions. The results of this study also provide a picture of the late kinetic steps more realistic than the simplified mechanism, which mimics Scheme 1 obtained in low temperature studies and which is commonly used for the interpretation of experimental results recorded under a variety of conditions. It is often ignored that while Scheme 1 may be a reasonable approximation at low temperatures under one set of conditions, it may not be adequate under other sets of conditions employed to selectively enhance specific intermediate forms. Low temperature and high pH is often preferred in rhodopsin studies to obtain specific information on the Meta I_{480} form and to track its conversion to different Meta II species.

In the current time-resolved optical absorption study on the photoconversion of bovine rhodopsin at 15 °C and at three pH values, pH 7.3, 8.0, and 8.7, we reinvestigate the late part of the kinetic scheme (Scheme 2) covering the microsecond to millisecond time window to determine whether the branched-square scheme is sufficient to fully describe the photokinetics of rhodopsin. The current study shows that a more complex extended branched-square scheme provides a better kinetic description. This information is essential for the deeper understanding of the later intermediates of rhodopsin leading up to the G-protein-activating Meta II state.

## MATERIALS AND METHODS

Hypotonically washed native bovine rhodopsin in membrane was prepared, and the membrane suspension was sonicated as previously described. For each experiment, 1–2 mg/mL of bovine rhodopsin was suspended in 100 mM NaCl solutions containing 10 mM BTP for pH 8.7 and 10 mM Tris for pH 8.0 and pH 7.3 experiments. Time-resolved optical absorption difference spectra, (post − pre)photolysis, in the 300–700 nm range were measured at 15 °C using an apparatus previously described. The difference spectra were collected at a series of delay times between 10 μs and 0.2 s following the ~7 ns excitation pulse of a 477 nm dye laser (80 μJ/mm²). Polarization of the probe light was set to 54.7° (magic angle) relative to the excitation pulse polarization to prevent absorbance changes due to the rotational diffusion of rhodopsin.

Prior to global exponential fitting, the data matrix, A, that represents the difference absorption spectra in the 330–650 nm range at all times was subjected to singular value decomposition (SVD): A = UΣV². Matrix U contains the orthonormal spectral vectors, V is the matrix of orthonormal temporal vectors, and the Σ matrix is a measure of the U and V vector pair contributions to the data matrix. The significant V vectors were fitted to a sum of exponential functions, and the b-spectra were calculated as described earlier. Microscopic reaction rate constants in the proposed kinetic schemes were obtained by fitting kinetic models to the b-spectra from the global exponential fit using the proposed spectra of the intermediates as described later. All calculations were performed by programs written in Matlab (The Mathworks Inc).

## RESULTS

Time-resolved absorption difference spectra, (post − pre)-photocexcitation, were recorded at 13 delay times of 10, 25, 50, 100, and 500 μs and 1, 2, 5, 10, 20, 50, 100, and 200 ms following laser excitation. The data taken at 15 °C sample temperature, normalized to the same protein concentration, are presented in the 330–650 nm wavelength range for pH 7.3, pH 8.0, and pH 8.7 in Figure 1, panels a, b and c, respectively. Arrows indicate the direction of the absorption change in time. In the microsecond time range, Lumi I and Lumi II, with absorption blue-shifted from that of the unphotolyzed (dark) pigment, are observed at all three pH values, and decay into a mixture of Meta I_{480} and deprotonated Meta forms seen in the millisecond time window. As expected, the highest Meta I_{480} content was evident at late times in the pH 8.7 sample, confirming the anomalous pH dependence of the equilibrium, as shown in Scheme 2, between the protonated Meta I_{380} and the deprotonated Meta II form.

### Global Exponential Fitting of Time-Resolved Spectral Data

Our kinetic analysis is based on the assumption that all the steps in the photoinduced reaction chain follow first order kinetics. The concentration changes of the intermediates in time can be described by a sum of time-dependent exponential functions. Thus, the absorption data matrix recorded in the experiment can be fit to a sum of exponential functions:

\[ A(\lambda, t) = \sum b_i(\lambda) \exp(-\alpha_i t) + D \]
denote wavelength and time, respectively, and of the residuals of the contributions to the absorption, called the states and the eigenvectors, \( \mathbf{E} \), the product of the intermediate spectra, present in the kinetic scheme. The matrix, which itself contains the microscopic rate constants of the reaction steps, and the reaction rate constants can be obtained by dissecting the absorption spectra of the intermediates in a unidirectional sequential scheme. This is the only scheme, their shapes showing the decay (positive components) and formation (negative components) of intermediates, which can be deduced by dissecting the absorption spectra of the intermediates in a unidirectional sequential scheme. This is the only scheme whose intermediates can be calculated from the experimental data without further assumptions. In the calculation, the apparent rates obtained in the exponential fit are used as the microscopic rate constants of the reaction steps, and the b-spectra are converted into the spectra of the sequential intermediates.\(^{16}\) The proposed mechanism can be tested and the reaction rate constants can be obtained by fitting models to the experimental b-spectra and lifetimes, as described later. Prior to the fitting, the spectra of the molecular states present in the proposed kinetic scheme have to be determined.

Deriving the Absorption Spectra of the Molecular States. The spectral shapes of the molecular intermediate states, including the LumI, LumII, Meta \( \text{I}_{380} \), and deprotonated Meta form absorbing at 380 nm (Meta \( \text{I}_{380} \)), which may be potentially present during the transitions following the photoexcitation of rhodopsin under the experimental conditions employed in this work, can be deduced by dissecting the absorption spectra of the intermediates in a unidirectional sequential scheme. This is the only scheme whose intermediates can be calculated from the experimental data without further assumptions. In the calculation, the apparent rates obtained in the exponential fit are used as the microscopic rate constants of the reaction steps, and the b-spectra are converted into the spectra of the sequential intermediates.\(^{16}\)

Because the b-spectra obtained for the pH 7.3 sample are very similar to the ones reported and interpreted mechanistically in earlier studies,\(^ {17,18} \) we use the spectra of the sequential

where \( r \), are the apparent rates, \( b(\lambda) \) are wavelength-dependent contributions to the absorption, called the b-spectra, \( \lambda \) and \( t \) denote wavelength and time, respectively, and \( D \) is the matrix of the residuals of the fit, which contains no information but experimental noise. Using SVD and fitting only a limited number of \( V \) vectors greatly simplify the global exponential fit. The data sets shown in Figure 1 for the three different pH values were adequately fitted with three exponentials with lifetimes of 24 \( \mu s \), 3.0 ms, and 36 ms for the pH 7.3; 25 \( \mu s \), 2.5 ms, and 27 ms for the pH 8.0; and 23 \( \mu s \), 2.4 ms, and 23 ms for the pH 8.7 samples. The reproducibility of lifetimes in multiple experiments was better than 20%. The b-spectra obtained in the fit, which represent the amplitudes of the exponential functions at all wavelengths, are shown for pH 7.3, 8.0, and 8.7 in Figure 2, panels a, b, and c, respectively. The residuals of the three-exponential fit, which are shown below the b-spectra for clarity, display only random noise. Because our primary interests are the millisecond processes, only a limited number of delay times were dedicated to obtain the absorption changes in the microsecond time window. This may have resulted in reduced accuracy of the first b-spectra and the corresponding lifetimes.

The b-spectra themselves have no direct physical meaning, for example, they cannot be regarded as difference spectra between intermediates. They merely represent spectral shapes with positive or negative amplitudes, present at zero time, which disappear as the exponential functions decay in time. The only exception is the b-spectrum corresponding to zero apparent rate, \( b_0 \), which represents the spectrum of the end products on the time scale of the experiment. The b-spectra, however, have direct mathematical connection to the kinetic matrix, which itself contains the microscopic rate constants present in the kinetic scheme. The b-spectra, \( \mathbf{b} \), are essentially the product of the intermediate spectra, \( \mathbf{E} \), of the molecular states and the eigenvectors, \( \mathbf{W} \), of the kinetic matrix, \( \mathbf{K} \):

\[
\mathbf{b} \langle \lambda, n \rangle = \mathbf{E} \langle \lambda, n \rangle \mathbf{W} \langle n, n \rangle
\]

Figure 1. (Post–pre)photoexcitation difference spectra recorded of rhodopsin membrane suspensions at 15 °C for (a) pH 7.3, (b) pH 8.0, and (c) pH 8.7 samples at 13 delay times, specified in the text, between 10 \( \mu s \) and 200 ms after laser excitation. The arrows indicate direction of spectral change as the delay time increases.

Figure 2. The b-spectra (amplitudes of exponential functions) and apparent lifetimes determined from the global exponential fit for (a) pH 7.3, (b) pH 8.0, and (c) pH 8.7 samples. The color of the lifetime presented matches the color of the corresponding b-spectrum: \( b_0 \), 23–25 \( \mu s \) (blue); \( b_1 \), 2.4–3.0 ms (green); \( b_2 \), 23–36 ms (red); \( b_3 \) (cyan). The residuals of the three-exponential fit are shown below the b-spectra for each pH.
intermediates at this pH to deduce the spectra of the molecular states. The absolute spectra shown in Figure 3a, solid lines,

were obtained by adding back the bleach spectrum. The spectrum of the bleached rhodopsin, which also contains isorhodopsin produced during the laser excitation, was deduced from analogous experiments carried out on an equivalent isorhodopsin sample solubilized in 0.2% dodecyl-maltoside detergent. The solubilized rhodopsin is known to produce only the Meta II form as the end product. The spectra of the sequential intermediates for pH 7.3 were reconstructed, Figure 3a dotted lines, as sums of the model spectra of the molecular states displayed in Figure 3b. The spectra of the protonated (Lumi I, Lumi II, Meta I480) and the deprotonated Meta molecular intermediate states are represented in blue, green, red, and cyan, respectively.

The absolute spectra of intermediates in the straight sequential scheme derived from the apparent lifetimes and experimental b-spectra of the pH 7.3 sample after the bleach was added back (solid lines). The reconstructed spectra of the sequential intermediates using the sum of model spectra are also shown (dotted lines). (b) The model spectra for the Lumi I, Lumi II, Meta I480, and the deprotonated Meta molecular intermediate states are represented in blue, green, red, and cyan, respectively.

Figure 3. (a) The absolute spectra of intermediates in the straight sequential scheme derived from the apparent lifetimes and experimental b-spectra of the pH 7.3 sample after the bleach was added back (solid lines). The reconstructed spectra of the sequential intermediates using the sum of model spectra are also shown (dotted lines). (b) The model spectra for the Lumi I, Lumi II, Meta I480, and the deprotonated Meta molecular intermediate states are represented in blue, green, red, and cyan, respectively.

The first sequential intermediate present on the early microsecond time scale is the well-known Lumi I form (blue lines in Figure 3a,b). It converts to Lumi II in a reversible reaction step with a 24 μs lifetime, in agreement with our earlier report.15,19 The Lumi II spectrum, Figure 3b, green line, was obtained from the second sequential intermediate spectrum, Figure 3a, green line, assuming an equilibrium constant of 4 for the Lumi I to Lumi II reversible step.15 As already mentioned, the first b-spectra and the corresponding lifetimes may be less accurate than the rest. However, this has no effect on our conclusions regarding the kinetics on longer time scales, which is the main focus of this report. The third and fourth sequential intermediates in Figure 3a (red and cyan curves, respectively) are well represented by mixtures of 0.86 + 0.14 and 0.43 + 0.57 of the Meta I480 and Meta II spectral forms presented in Figure 3b (red and cyan curves, respectively).

Often kinetics are tentatively assumed to follow unidirectional sequential schemes. In our case, the sequential intermediates contain more than one molecular state and thus this simple mechanism does not describe the kinetics properly. Despite its limitations and shortcomings, the sequential mechanism with intermediates derived from low temperature studies, is the most frequently cited one under all kinds of experimental conditions.

The Square Model Is Not Sufficient to Interpret the Experimental b-Spectra. The square model, Scheme 2, previously proved to accurately describe experimental data under a variety of experimental conditions.14,16–18 The reaction rate constants in the kinetic scheme are obtained by a fitting procedure. During the fitting the microscopic rate constants in the kinetic matrix are adjusted, the eigenvalues and eigenvectors of the kinetic matrix are calculated, and finally, the eigenvalues are compared with the experimental apparent rates and the reproduced b-spectra to the experimental b-spectra obtained in the global exponential fit.

Before executing this fitting strategy, we have to deal with one problem. The number of exponentials found in the global fit to our experimental data is three, and thus we have four b-spectra, one less than predicted by the mechanism incorporated in the square model. This is in agreement with earlier temperature dependence studies at pH 7.4,18 which showed that the b-spectrum associated with the Lumi to Meta I380 reversible conversion, and occurring with a lifetime of around 100 μs at room temperature, becomes undetectable at 15 °C. This is because the equilibrium is very back-shifted at lower temperatures and shows practically no net conversion. Rather than simply eliminate the poorly populated Meta I380 state from the scheme, thus blocking the reaction flow through that state, we circumvent the problem by adding a b-spectrum of negligible amplitude to the four b-spectra found in the exponential fit. It represents the Lumi II to Meta I380 transition and has an apparent lifetime of 250 μs based on the reported temperature dependence.19 With this addition, we have a total of four lifetimes and five b-spectra, which we call experimental to distinguish them from the ones reproduced by the kinetic model in our analysis.Lifetime1 is 24, 25, and 23 μs, lifetime2 is set to 250 μs, lifetime3 is 3.0, 2.5, and 2.4 ms, and lifetime4 is 36, 27, and 23 ms for pH 7.3, 8.0 and 8.7, respectively.

The results of the kinetic fit (dots) to the b-spectra (solid lines) are shown for pH 7.3, pH 8.0, and pH 8.7 in Figure 4, panels a, b, and c, respectively. The fit for the pH 7.3 sample is nearly perfect, and the fit to the b-spectra of the pH 8.0 sample is still acceptable, considering the level of experimental noise and reproducibility of spectral shapes. We failed to reproduce the b-spectra for the pH 8.7 sample despite our numerous attempts, which included slight modifications to the λmax and the amplitudes of the model spectra. The problems associated with this fit go beyond small adjustments to the model spectra and point toward a kinetic mechanism more complex than the one represented by the square model.

Extending the Square Model. Analysis of b-Spectra and Identifying the Intermediates Present in the Kinetics. The failure to find an acceptable fit to the b-spectra for the pH 8.7 sample at 15 °C suggests that at lower temperatures and elevated pH values the observed kinetics is even more complex than the one described by the square model. Most of the information on the kinetic mechanism that is likely to produce a better fit to the experimental b-spectra can be obtained from the b-spectra themselves.

As seen in Figure 4, the first b-spectra, blue lines, with lifetimes of 23–25 μs at all three pH values studied show the partial conversion of Lumi I to Lumi II. The second b-spectra, green lines, with lifetimes of 250 μs were introduced by us, as
described above, and represent a negligible conversion of the Lumi I/Lumi II mixture into the Meta I480 form. The third b-spectra, red lines, with lifetimes of 2.4-3.0 ms are consistent with the transition of this mixture into the Meta I480 form with very small simultaneous Meta II formation. The amplitude of the third b-spectrum for the pH 8.7 sample is somewhat smaller than the rest, which can be interpreted as a partial conversion of the Lumi I/Lumi II mixture into the Meta I480 state. As expected, the fit produced by the square model to this experimental b-spectrum has a matching shape but is of bigger amplitude, showing the spectral change of the full conversion predicted by the square model. The fourth b-spectra, cyan lines, with lifetimes of 23-36 ms deserve special attention and will be discussed in detail below. The end products seen in the experimental b-spectra, magenta lines, can be reconstructed at all three pH values using mixtures of the Meta I480 and Meta II forms, minus the bleached rhodopsin, and do not require the presence of any additional spectral forms.

The shape of the fourth b-spectrum for the pH 8.7 sample could not be fit with the square model using the spectra of the molecular states derived from the spectra of the sequential intermediates at pH 7.3. Figure 5a shows that there is a substantial difference in shape between this b-spectrum, red curve, and that of the pH 7.3 sample, blue curve, and it is somewhat less for the pH 8.0 sample, green curve. The b-spectra shown in the figure were normalized to have the same amplitude for easier comparison. The b-spectrum for the pH 7.3 sample is consistent with the conversion of Meta I480 to Meta II as predicted by the square model. The b-spectrum for the pH 8.0 and pH 8.7 samples do not match the spectral shape corresponding to this conversion and their interpretation is more problematic. The suggestion that the Meta I480 spectrum is simply shifted to the red at high pH and low temperature is not viable because the corresponding b-spectrum of the solubilized rhodopsin sample at pH 8.7, recorded at 15 °C temperature, does not require a shifted Meta I spectrum and follows the normal Meta I480 to Meta II transition (Figure 5a, cyan curve). In order to understand the transition represented by the fourth b-spectrum of the pH 8.7 sample, we plotted the difference between the normalized b-spectra of the pH 8.7 and pH 7.3 samples shown in Figure 5a and compared it with the 2.4 ms experimental b-spectrum for the pH 8.7 sample, Figure 5b, blue and green curves, respectively. The third b-spectrum was interpreted above as the transition of the 4:1 mixture of Lumi II and Lumi I into Meta I480 with little if any Meta I480 formation. The difference spectrum and the 2.4 ms b-spectrum have very similar shapes, suggesting that Meta I480 may form not only in the early millisecond step but also in the late millisecond step. This would suggest two Meta I480 forms present in the kinetics, the first one formed with 2.4 ms lifetime from the Lumi I + Lumi II mixture and the second one formed from the first one with 23 ms lifetime at pH 8.7.

The square model cannot describe this complex picture. When Meta I480 formation from Lumi is negligible because this reversible step has a very back-shifted equilibrium, as is the case at all pH values in our experiment, the square model predicts a complete Meta I480 formation in a single transition, which in our case would occur with the early millisecond lifetime. This is seen at pH 7.3, and to some extent even at pH 8.0. The pH 8.7 sample also shows Meta I480 formation with 2.4 ms lifetime. However, the reduced amplitude of the b-spectrum shows that it is not complete. Incomplete Meta I480 formation from Lumi II means that this step is reversible. Introducing a reversible Meta I480 formation into the square model alone does not solve the problem. It helps match the reduced amplitude of the 2.4 ms b-spectrum, but it does not explain the fraction of Meta I480 formed later with a 23 ms lifetime. In addition, it also predicts the presence of a Lumi I + Lumi II mixture in the end products, which under our experimental conditions are adequately reproduced by a mixture of the Meta I480 and Meta II forms alone.

**Proposing the Double-Square Model.** The reduced amplitude of the experimental b-spectrum with 2.4 ms lifetime, the two-step formation of Meta I480 and the absence of the Lumi I + Lumi II mixture in the end products at pH 8.7 suggest a kinetic model that contains two isospectral Meta I480 states as shown in Scheme 3. The first, Meta I480 state is formed from Lumi II via a reversible step and converts into the second, Meta

Figure 5. (a) Comparison of the late-millisecond experimental b-spectra for samples at pH 8.7 (red), pH 7.3 (blue), and pH 8.0 (green) and detergent solubilized sample at pH 8.7 (cyan) at 15 °C normalized to the same amplitude. (b) Comparison of the difference between the normalized late-millisecond b-spectra for pH 8.7 and pH 7.3 samples (blue) and the 2.4 ms b-spectrum for the pH 8.7 sample (green).
Scheme 3. New Double-Square Activation Mechanism Scheme with the Earlier Intermediates in Gray and the Later Intermediates, Which Were the Focus of This Study, in Black

The restrictions regarding the values of the microscopic rate constants are more severe in case of temporal than spectral degeneracy. In the latter case, the kinetic matrix may have one or two unrestricted lifetimes, which have close to zero spectral amplitude due to the presence of isospectral intermediates. This can lead to multiple solutions and a number of submodels with different sets of microscopic rate constants. Our goal is to demonstrate the feasibility of degeneracy cases rather than explore the range of all possible solutions.

**Submodels with Temporal Degeneracy.** It is far easier to deal with temporal degeneracy, the degeneracy of the kinetic matrix, than with spectral degeneracy. Note that we have the case of double degeneracy when the double-square model is fit to the four experimental lifetimes and five b-spectra. Moreover, the degenerate lifetimes must be selected prior to fitting, which makes the fit biased by the selection. One of the two degeneracies can be assigned to the late millisecond experimental lifetime, lifetime4 of 23 ms, because extension of the kinetic scheme by adding a second square to it replaces the Meta Ia480 to Meta II final step in the square model with a superposition of processes in the double-square model. Either of the two millisecond experimental lifetimes (lifetimes 3 of 2.4 ms and lifetime4 of 23 ms) are candidates for the second degeneracy.

We explored both of these possibilities during the fitting of the double-square model to the experimental b-spectra and lifetimes of the pH 8.7 sample. The spectra of the intermediate states used in the fit were the ones derived from the spectra of the pH-7.3 sequential intermediates, as described above, with the exception of the Meta Ib480 state. The spectrum assigned to this state is ~2 nm blue-shifted from the Meta Ia480 spectrum and has also ~3% higher amplitude, which indicates that a spectral shift is also part of the 23 ms b-spectrum of the pH 8.7 sample. The results of the fit when both the 2.4- and 23 ms lifetimes are considered degenerate, degen(3,4;5,6), are shown in Figure 6a where the experimental b-spectra are compared with the ones reproduced by the kinetic matrix of the double-square model. To achieve this type of degeneracy in the fit, the eigenvalues 3 and 4 of the fitted kinetic matrix are forced to merge and match the early millisecond lifetime. Following the events seen in the single-square model, the early, 2.4 ms lifetime corresponds to the formation of the Meta Ia480 and Meta Ila states, and now it also includes the reversible conversion between them. The eigenvalues 5 and 6 of the fitted kinetic matrix are also forced to merge and match the late, 23 ms, lifetime, which is associated with the formation of the final Meta Ib480 and Meta IIb states. The degenerate eigenvectors corresponding to the merged eigenvalues are summed up before the reproduced b-spectra are calculated. Note that in practice we achieve only quasi- and not true degeneracy, and the term merging means to have similar but not necessary identical values of the lifetimes. The microscopic rate constants in the fitted kinetic matrix are listed in Table 1, column 1. As mentioned above, there are multiple solutions to the kinetics. Therefore, it is the range rather than the exact value of the microscopic rate constants that has physical significance. It is

Figure 6. Comparison of the experimental b-spectra (solid lines) and the b-spectra reproduced by the extended double-square scheme (dots) for b-spectra b1 (blue), b2 (green), b3 (red), and b4 (cyan) for pH 8.7. (a) Temporal degeneracy case: both 2.4- and 23 ms lifetimes are considered degenerate. (b) Spectral degeneracy case: reproduced lifetimes of 6.7 and 10 ms correspond to b-spectra with negligible amplitudes.
While it was possible to reproduce the lifetimes and combination, the product of the eigenvector and the lifetime is considered degenerate, $\text{degen}(4,5,6)$. In this type of each process that are the primary concern of this study.

Two Meta II Forms

| Table 1. Microscopic Reaction Rate Constants in the Double-Square Model with Temporal Degeneracy |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| pH 8.7 $k/\overline{k}$                  | pH 8.0 $k/\overline{k}$                  | pH 7.3 $k/\overline{k}$                  |
| Lumi I $\leftrightarrow$ Lumi II         | $3.5 \times 10^{3.7}/7.7 \times 10^{3}$  | $3.4 \times 10^{3.7}/7.7 \times 10^{3}$  |
| Lumi II $\leftrightarrow$ Meta I$_{580}$  | $1.1 \times 10^{2}/2.4 \times 10^{1}$    | $1.2 \times 10^{2}/1.9 \times 10^{1}$    |
| Lumi II $\leftrightarrow$ Meta I$_{480}$  | $4.1 \times 10^{2}/4.7 \times 10^{1}$    | $3.8 \times 10^{2}/5.3 \times 10^{1}$    |
| Meta I$_{480}$ $\leftrightarrow$ Meta Ila | $1.5 \times 10^{2}/5.2 \times 10^{1}$    | $2.0 \times 10^{2}/5.8 \times 10^{1}$    |
| Meta Ia$_{480}$ $\rightarrow$ Meta Ila   | $3.4 \times 10^{2}/2.3 \times 10^{1}$    | $5.9 \times 10^{2}/2.0 \times 10^{1}$    |
| Meta Ia$_{480}$ $\rightarrow$ Meta IIa   | $3.9 \times 10^{3}/3.9 \times 10^{2}$    | $2.9 \times 10^{3}/2.9 \times 10^{2}$    |
| Meta Ib$_{480}$ $\rightarrow$ Meta Ila   | $1.2 \times 10^{2}/3.1 \times 10^{1}$    | $1.7 \times 10^{2}/2.0 \times 10^{1}$    |
| Meta Ila $\rightarrow$ Meta IIa          | $1.4 \times 10^{2}/5.3 \times 10^{1}$    | $1.1 \times 10^{2}/3.8 \times 10^{1}$    |

| Table 2. Microscopic Reaction Rate Constants in the Double-Square Model with Spectral Degeneracy |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| pH 8.7 $k/\overline{k}$                  | pH 8.0 $k/\overline{k}$                  | pH 7.3 $k/\overline{k}$                  |
| Lumi I $\leftrightarrow$ Lumi II         | $3.5 \times 10^{3}/7.5 \times 10^{2}$    | $3.3 \times 10^{3}/7.7 \times 10^{2}$    |
| Lumi II $\leftrightarrow$ Meta I$_{580}$  | $1.1 \times 10^{2}/2.3 \times 10^{1}$    | $1.3 \times 10^{2}/2.1 \times 10^{1}$    |
| Lumi II $\leftrightarrow$ Meta I$_{480}$  | $3.8 \times 10^{2}/6.0 \times 10^{1}$    | $3.4 \times 10^{2}/4.2 \times 10^{1}$    |
| Meta I$_{480}$ $\leftrightarrow$ Meta Ila | $1.7 \times 10^{2}/1.7 \times 10^{1}$    | $1.9 \times 10^{2}/1.8 \times 10^{1}$    |
| Meta Ia$_{480}$ $\rightarrow$ Meta IIa   | $8.7/11.2$                                | $1.3 \times 10^{2}/1.1 \times 10^{1}$    |
| Meta Ia$_{480}$ $\rightarrow$ Meta Ib$_{480}$ | $4.0 \times 10^{2}/4.0 \times 10^{1}$    | $3.3 \times 10^{2}/3.3 \times 10^{1}$    |
| Meta Ib$_{480}$ $\rightarrow$ Meta IIb   | $2.8 \times 10^{2}/6.8 \times 10^{1}$    | $4.8 \times 10^{2}/5.8 \times 10^{1}$    |
| Meta Ila $\rightarrow$ Meta IIb          | $1.3 \times 10^{2}/2.5 \times 10^{2}$    | $1.2 \times 10^{2}/1.2 \times 10^{2}$    |

| Table 3. Microscopic Reaction Rate Constants in the Double-Square Model with Spectral Degeneracy Submodel Ending with Two Meta II Forms |
|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| pH 8.7 $k/\overline{k}$                  | pH 8.0 $k/\overline{k}$                  | pH 7.3 $k/\overline{k}$                  |
| Lumi I $\leftrightarrow$ Lumi II         | $3.6 \times 10^{3}/7.8 \times 10^{2}$    | $3.4 \times 10^{3}/7.7 \times 10^{2}$    |
| Lumi II $\leftrightarrow$ Meta I$_{580}$  | $1.1 \times 10^{2}/2.4 \times 10^{1}$    | $1.3 \times 10^{2}/2.0 \times 10^{1}$    |
| Lumi II $\leftrightarrow$ Meta I$_{480}$  | $4.1 \times 10^{2}/5.2 \times 10^{1}$    | $3.8 \times 10^{2}/3.2 \times 10^{1}$    |
| Meta I$_{480}$ $\leftrightarrow$ Meta Ila | $1.7 \times 10^{2}/6.8 \times 10^{1}$    | $2.0 \times 10^{2}/4.0 \times 10^{1}$    |
| Meta Ia$_{480}$ $\rightarrow$ Meta IIa   | $5.3/3.5 \times 10^{3}$                   | $1.0 \times 10^{2}/3.7 \times 10^{3}$    |
| Meta Ia$_{480}$ $\rightarrow$ Meta Ib$_{480}$ | $3.8 \times 10^{2}/3.8 \times 10^{3}$    | $2.6 \times 10^{2}/2.6 \times 10^{3}$    |
| Meta Ib$_{480}$ $\rightarrow$ Meta IIb   | $8.8/3.1 \times 10^{2}$                   | $1.2 \times 10^{2}/2.1 \times 10^{2}$    |
| Meta Ila $\rightarrow$ Meta IIb          | $9.0 \times 10^{2}/4.9 \times 10^{2}$    | $8.0 \times 10^{2}/3.9 \times 10^{2}$    |

the overall mechanism rather than the specific rates assigned to each process that are the primary concern of this study.

In the other case of temporal degeneracy only the late, 23 ms, lifetime is considered degenerate, $\text{degen}(4,5,6)$. In this type of degeneracy, the eigenvalues 4, 5, and 6 of the fitted kinetic matrix are forced to merge and match the late millisecond lifetime. Physically, it means slowing the conversion between the Meta I$_{480}$ and Meta IIa states and forcing all the apparent transitions produced by the lower cycle to occur with a single late millisecond lifetime. The rate restrictions in this case are so severe that none of our attempts produced acceptable results. While it was possible to reproduce the lifetimes and $b$-spectra, each within an acceptable range of error, the experimental data matrix itself could not be reproduced adequately. This is because reproduction of $b$-spectra and lifetimes is only approximate and considers the eigenvectors and eigenvalues of the kinetic matrix separately, while reproduction of the data matrix, though also approximate, deals with their mathematical combination, the product of the eigenvector and the exponential of the corresponding eigenvalue.

Submodels with Spectral Degeneracy. We also tested the case of spectral degeneracy. Out of the six lifetimes and seven $b$-spectra produced by the kinetic matrix, four lifetimes and five $b$-spectra were forced to match the four experimental lifetimes and five experimental $b$-spectra. The remaining two lifetimes of the kinetic matrix were unrestricted. However, their corresponding $b$-spectra were forced to have amplitudes below detection limits.

The results of the fit for the pH 8.7 sample in the case of spectral degeneracy are shown in Figure 6b, where the experimental $b$-spectra are compared with the ones reproduced by the kinetic matrix. The fit is as good as that seen for the temporal degeneracy above. The two unrestricted lifetimes, 6.7 and 10 ms, corresponding to the $b$-spectra with negligible amplitudes produced by the fit, have similar values and are close to being degenerate. The microscopic rate constants are listed in Table 2, column 1.

Spectral Degeneracy Submodel Ending with Two Meta II Forms. A common characteristic of the submodels discussed so far is the irreversibility of the Meta Ib$_{480}$ and Meta IIb formation steps leading to a final stage that is composed of these two states only. There is, however, a different rate combination in the double-square model with spectral degeneracy that is also consistent with the experimental results. In this version, both the formation of Meta Ib$_{480}$ and Meta IIb from Meta Ia$_{480}$ are irreversible. The two Meta II states, Meta IIa and Meta IIb, are in equilibrium with comparable forward and backward rate constants and thus not only Meta IIb but Meta IIa too is part of the final composition, together with Meta Ia$_{480}$. The two unrestricted apparent lifetimes, destined to have insignificant amplitudes, produced by this version of the fit are 6.6 and 38 ms. One lifetime is shorter while the other is longer than the late millisecond restricted lifetime, as opposed
to the values mentioned for the previous submodel, which were both between the early and late millisecond restricted lifetimes. The microscopic rate constants are in Table 3, column 1.

Temporal and Spectral Degeneracy Models for the pH 7.3 and 8.0 Samples. The fitting strategy worked out for the pH 8.7 sample, and described in detail above, was repeated for the pH 7.3 and pH 8.0 samples. The microscopic rate constants obtained in the fit, including both temporal and spectral degeneracy cases, are presented in Tables 1 and 2, columns 3 and 2, for pH 7.3 and 8.0, respectively. As already discussed above, in the spectral degeneracy case two lifetimes of the kinetic matrix were unrestricted, not forced to match any of the four experimental lifetimes. These lifetimes were similar and close to being degenerate, 7.2 and 9.7 ms for pH 7.3, and 7.1 and 9.4 ms for pH 8.0. The variation of the spectral degeneracy model ending with Meta Ila and Meta IIb states in equilibrium was also obtained for the pH 7.3 and 8.0 samples and produced 12 and 46 ms and 7.9 and 42 ms unrestricted lifetimes, respectively, with negligible b-spectra amplitudes. The microscopic rate constants are in Table 3, columns 3 and 2 for pH 7.3 and pH 8.0, respectively.

■ DISCUSSION

The most important result of the analysis presented above is that the rhodopsin kinetics recorded at moderately high pH and low temperature could not be described by the square model and required a more complex one that we refer to as the double-square model. It is plausible to assume that the same double-square model applies also at neutral and even slightly acidic pH values, and it is merely a technical question whether the model can or cannot be recognized experimentally. In our UV–vis absorption experiments the reversible and incomplete conversion of the Lumi I/Lumi II mixture into Meta Ia480 at pH 8.7 was the key observation leading to the model. When this reversible step is very forward shifted, the only spectral signature the entire lower square of the model produces is a difference spectrum. The same difference spectrum can also be produced by a single step, the reversible conversion of Meta Ia into Meta II in the original square model. This is the reason why the square-square model proved to be adequate to describe the UV–vis absorption changes at lower pH values. When the double-square model is fit to the experimental b-spectra and lifetimes of the pH 7.3 sample, the fit does not require reversible Lumi II to Meta Ia480 step. At pH 8.0 and 8.7, however, it is needed in order to produce acceptable results.

Time Evolution of Intermediate States. The rise and fall of intermediates in the double-square model is best visualized by plotting the intermediate concentrations as a function of time. The concentrations are calculated based on the microscopic rate constants of the kinetic matrix using the eigenvector matrix and the matrix of the time-dependent exponential functions of the eigenvalues.16 The time evolution of the seven intermediates for the temporal and spectral degeneracy submodels, with the exception of the one ending with two Meta II states, are practically the same. The concentration profiles for the temporal degeneracy, degen-(3,4;5,6), are shown in Figure 7, panels a, b and c for pH 7.3, 8.0, and 8.7, respectively, where the dots indicate the actual experimental measurement times. There is hardly any difference among the three pHs at early times because the equilibrium constant in the Lumi I to Lumi II reversible step was fixed by us. At later times, a small fraction of the equilibrated Lumi I/Lumi II mixture is seen lingering at pH 8.0 and 8.7 as compared with pH 7.3 due to the reversible Meta Ia480 formation. This difference among the three pHs, which looks almost insignificant in this representation, played a very important role in proposing the double-square model. Because of its small relative size, it does not cause a big difference between the time profiles of the Meta Ia480 concentration at the different pHs, in accord with the similar amplitudes of the early millisecond b-spectra, which account for the formation of this state. As expected, the ratio of the Meta IIb480 to Meta IIb concentrations increases at higher pH values. For comparison, panel a also shows the time evolution of the intermediate states in the square model for the pH 7.3 sample. It is seen that the double-square model simply splits each of the Meta Ia480 and Meta II states of the square model into two consecutive states. The Meta Ia480 state forms the same way as the Meta Ia480 state does in the square model. However, it no longer stays stagnant but converts into the next, and also different, Meta IIb state in which the Schiff base has an enhanced ability to undergo deprotonation. The fraction of the deprotonated form is much lower at the Meta Ia480 stage, Meta IIa, than at the final Meta IIb480 stage, Meta IIb, where it becomes very pH dependent, and at pH 7.3, it is higher than the Meta IIb480 fraction. Deprotonation of the Schiff base itself is likely to be a fast reaction step because under favorable conditions it is observed even in the Lumi state on the hundred microsecond time scale (160 μs for the solubilized sample under our experimental conditions). In the double-square model, the Schiff base deprotonates simultaneously with the formation of the Meta IIb480 state, as opposed to the square model where deprotonation is seemingly detached from any other transition and occurs long after the Meta Ia480 state is formed with ten times faster rate. It is intriguing to relate the
formulation of Meta Ib$_{480}$ and the enhanced deprotonation leading to the formation of Meta Ib to the helix-6 movement observed by EPR on a similar time scale. 28,31 This helix movement is necessary for G-protein binding. It is also suggested that the proton uptake by the protein, leading to its active state and causing the anomalous pH dependence of the equilibrium between Meta I and Meta II in Scheme 1, is initiated by the helix movement. 21–23,25–27 Whether proton uptake is an additional step or part of the late steps in our scheme cannot be answered at this time and requires further studies.

The concentration time dependence for the intermediate states of the spectral degeneracy submodel ending with two Meta II states is presented in Figure 8, panels a, b, and c for pH 7.3, 8.0, and 8.7, respectively. It shows the prolonged presence of Meta IIa at the expense of Meta Ib. The rest of the states follow the same time dependence pattern seen for the other degeneracies.

The Physical Meaning of Degeneracy Submodels. Despite the differences in the values of the microscopic rate constants between the temporal and spectral degeneracy submodels, they produce the same concentration time dependence of intermediate states within uncertainty of the fit. The different submodels are merely variations of the same reaction network given by the double-square model and differ only in the flow rates of the individual supply and drain lines as determined by the microscopic rate constants. The submodels all possess the same combined flow capacities in the reaction steps in order to maintain the same buildup and decay time profiles of intermediates in the scheme. This can be done in more than one way because there are multiple reaction paths that are utilized for building up or reducing the concentrations of the intermediates in the scheme. When there are multiple supply lines to build up the concentration of an intermediate, the combined input flow is shared between them, and it can be accomplished in many ways for a single intermediate. The same applies to reducing the intermediate concentration by the drain lines. However, there are only a limited number of ways to do that for all intermediates simultaneously because the supply and drain lines are interwoven in a complex way; inflow for one intermediate is outflow for the other one.

The presence of reversible steps also contributed to the complexity of the double-square model. Because no Lumi states were detected in the final product, the Meta Ia to Meta Ib step was taken to be irreversible at all pHs. An additional step, the Lumi II to Meta Ia, was also set irreversible at pH 7.3. Note that the term irreversible means irreversible in practice, and these irreversible steps in the scheme are reversible in the mathematical sense with back rates set 10^4 times slower than the forward rates to ensure that the back rate has no influence on the results. The other rate constants in the scheme were unrestricted. The only restriction applied was one required for a closed cycle reaction path. In Tables 1 and 2 the Meta IIa to Meta Ib step is practically irreversible at all three pHs, and the Meta Ib$_{380}$ to Meta IIa step is only slightly reversible. Thus, the Meta Ib$_{380}$ + Meta I Ib end product is well separated from the earlier intermediates.

Because only the Meta Ia to Meta Ib step was set irreversible in the lower square, and the Meta IIa and Meta I Ib are isospectral states indistinguishable by our technique, the submodel ending with Meta IIa + Meta I Ib emerged. As seen in Table 3, the Meta Ib$_{480}$ + Meta IIa + Meta I Ib end product is well separated by irreversible steps not only from Meta Ia$_{480}$ but also from Meta Ib$_{380}$.

Under conditions different from ours, the end product may also contain the Lumi form 30,31 and the Meta Ia to Meta Ib step can no longer be considered irreversible. In that case the reaction chain in the double-square model may end with not only two Meta II states but also two Meta I states. Thus, the final steps in our new double-square scheme are consistent with the scheme deduced from G-protein peptide mimicking studies. 30,31 That scheme involved equilibria between late intermediates. Since that study was carried out at 0 °C and with detergent solubilized samples, while this study used 15 °C membrane samples, it is possible that these transitions involve equilibria that are more back-shifted in detergent solubilized samples at 0 °C than in membrane samples at 15 °C. The double-square model reveals the complexity of rhodopsin activation and how the activation mechanism varies depending on conditions. Recent structural studies show complex helix movements during activation of rhodopsin, which involve various H-bond rearrangements. 21–27 Crystal structures and solid-state NMR structures suggest a large movement in the helix as well as chromophore relaxation during the Meta I intermediate, which could lead to the two isospectral Meta I intermediates observed. 21,26 The structural studies of rhodopsin intermediates have used low temperatures to trap specific intermediates whose structures are studied. Just as with early optical studies of rhodopsin photolysis intermediates, it is important to remember that only a subset of the photolysis intermediates can be trapped at low temperatures. It is thus not possible to identify specific intermediates observed in this work with specific structures observed by others. To do that will require time-resolved structural studies under environmental conditions similar to these time-resolved spectral studies.

Under physiological conditions, some of the intermediates in the double-square model will be short-lived and the intermediates may not accumulate enough to make them

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**Figure 8.** Time-dependent concentrations between 1 μs and 1 s of the seven intermediates: Lumi I (blue), Lumi II (green), Meta I$_{380}$ (red), Meta Ia$_{480}$ (cyan), Meta IIa (magenta), Meta Ib$_{480}$ (orange), and Meta IIb (black) for the double-square model in the case of spectral degeneracy ending with two Meta II forms for samples at (a) pH 7.3, (b) pH 8.0, and (c) pH 8.7.
The kinetics of bovine rhodopsin in native membrane environment at moderately low temperature, 15 °C, and alkaline pH cannot be adequately described by the square model. A more complex double-square model is introduced and evaluated at pH 7.3, 8.0, and 8.7 by fitting it to the experimental data. Multiple submodels representing temporal and spectral degeneracies, which contain different sets of rate constants for the individual transitions within the double square scheme, are found to describe the UV–vis experimental data equally well. To distinguish between the different submodels will require other, more structure sensitive time-resolved spectral techniques. The scheme presented here, however, is consistent with EPR studies describing the helix-6 movement involved in activation, and G-protein peptide mimic binding studies. Many structural studies reveal complex helix movements involving the rearrangements of H-bonding in the transmembrane region of the protein, and several states of the chromophore relaxation during activation. Since the structural studies that reveal those motions are carried out by trapping intermediates at low temperatures and only some of the intermediates are likely to be trapped at low temperature, it is not possible to identify specific structures with specific intermediates observed here. Such correlations will require time-resolved structural studies under conditions that are comparable to photolysis conditions used in this study. The double-square model depicts the complexity of rhodopsin activation in membranes. The alternative pathways shown in the general scheme provide an important and useful background for future time-resolved structural studies of rhodopsin activation.

Some of the kinetic steps revealed under our conditions, although still part of the molecular mechanism, may appear obscured under physiological conditions of temperature and pH where some of the intermediates observed here would not accumulate at observable concentrations. The formal mechanistic scheme representing the dominant path of the complex activation mechanism in Scheme 3 may then appear fairly simple. However, it is important to understand the relationships of all of the intermediates seen here so that as other studies of rhodopsin are carried out under different pH and temperature conditions, in order to enhance the contributions of different intermediates, researchers can have a better understanding of the presence and transformations of the intermediates under study.

CONCLUSIONS

The presence and transformations of the intermediates under conditions, in order to enhance the contributions of di-rhodopsin are carried out under di of all of the intermediates seen here so that as other studies of intermediates accumulate at observable concentrations. The formal mechanism, although still part of the molecular mechanism, may appear complex. The alternative pathways shown in the double-square model depicts the complexity of rhodopsin activation, comparable to photolysis conditions used in this study. The time-resolved structural studies under conditions that are physiological, however, are likely to be trapped at low temperature, it is not possible to identify specific structures with specific intermediates observed here. Such correlations will require time-resolved structural studies under conditions that are comparable to photolysis conditions used in this study. The double-square model depicts the complexity of rhodopsin activation in membranes. The alternative pathways shown in the general scheme provide an important and useful background for future time-resolved structural studies of rhodopsin activation.

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ABBREVIATIONS

GPCR, G-protein coupled receptor; Rho, rhodopsin; Batho, bathorhodopsin; BSI, blue-shifted intermediate; Lumi, lumirhodopsin; Meta, metarhodopsin; BTP, 1,3-bis[(tris-(hydroxymethyl)-methylamino) propane; Tris, (tris(hydroxymethyl)methyl)aminoethane; SVD, singular value decomposition

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