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D96N Mutant Bacteriorhodopsin Immobilized in Sol-Gel Glass Characterization

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ABSTRACT

The D96N mutant form of bacteriorhodopsin (BR) purple membrane fragments isolated from the bacterium *Halobacterium salinarium* has been immobilized by entrapment in sol-gel glass. The protein was characterized for M state decay rate at different temperatures and pH values. Bleaching efficiency and absorbance maxima vs pH were also determined. The kinetic effects of triethanolamine and diethanolamine were also examined. Results indicated that the immobilized BR was affected in a manner similar to the mutant BR in aqueous suspension. Addition of guanidine, however, caused the immobilized BR to show kinetic parameters more closely related to the wild-type protein than the D96N mutant control. Samples of the aqueous suspension were characterized for particle size and particle size distribution. Dried samples of the immobilized BR were analyzed by field emission microscopy and BET to characterize both the purple membrane fragments and the sol-gel pore characteristics.

Index Entries: Immobilized; bacteriorhodopsin; D96N mutant; sol-gel; bleaching efficiency; light-sensitive protein.

INTRODUCTION

Sol-gel procedures are now available for the entrapment of enzymes and various other macromolecules in optically transparent silicate glasses (*1–3*). These entrapped proteins retain their optical (*1,3*) and biological

properties (2–5). Sol-gel immobilization has recently been successfully utilized for entrapment of both the native (3) and D96N mutant (4) form of bacteriorhodopsin (BR).

Bacteriorhodopsin and other light-sensitive proteins are of great interest because of their potential usefulness in photovoltaic devices, photoimaging, and molecular computing (*6–19*). A very recent article discussed the application of BR for an artificial retina (*20*).

Bacteriorhodopsin is the protein of the purple membrane of *Halobacterium salinarium*. It is a molecule of mol wt 26,000 Dalton consisting of 248 amino acid residues strung into seven trans-membrane α-helices. This protein acts as a light-driven proton pump that transports H^+ ions from the cytoplasm to the extracellular space with high efficiency (*21*). The proton pumping results in a pH gradient that is used by the organism for the synthesis of ATP from ADP. The photocycle consists of a series of steps that are initiated on absorption of a photon. During the photocycle, the proton translocation is accomplished by deprotonation of a Shiff base and its subsequent reprotonation. The deprotonation step creates the M state intermediate; the reprotonation initiates the M state decay. During this process, the absorption maximum shifts from approx 570 to 410 nm and then back to 570 nm on reaching the ground state from which the cycle was initiated. The M state can be divided into two intermediate states M^I and M^{II}. Since these intermediate states are indistinguishable under our experimental conditions, we can consider the two states together as the M state. The photocycle is actually complex. However, it is known that the proton from the Shiff base is released to the amino acid Asp85. The reprotonation step is catalyzed by Asp96.

There are several known variants of BR with modified optical properties. These have been prepared by either exchange of the retinylidene residue for a retinal analog molecule (*22–24*) or by modification of the amino acid sequence of the BR (*25,26*). One particular mutant with slowed M state decay has been described (*27*). This D96N mutant differs from the wild type by exchange of Asp96 for Asn. Thus, the internal proton donor capabilities are lost, and the M decay depends on the extracellular pH. The result of this amino acid exchange is manifested in the prolonged lifetime of the M state by several orders of magnitude compared with the wild type at alkaline pH values. The wild type (*3*) and the D96N mutant (*4*) have been previously found capable of entrapment in a sol-gel glass. The present article on the D96N mutant entrapped in sol-gel glass further characterizes this adduct. We have examined the kinetics, pH effects, thermal characteristics, and the effect of several additives that are known to decrease the M state decay rate in the wild-type BR (*28–30*). In addition, we have examined the physical characteristics of the dried glass for pore radius, pore volume, and pore surface area.

MATERIALS AND METHODS

Bacteriorhodopsin used in this study was supplied by A. B. Druzhko, Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino (Moscow Region), Russia. Tetramethoxysilane (TMOS) was purchased from Aldrich Chemical Company, Milwaukee, WI. All other chemicals were reagent-grade.**

Preparation of Sol-Gel D96N BR Containing Glass

The sol-gel glass was prepared as previously described (*4*). Seven milliliters of TMOS, 3.0 mL distilled water, and 0.1 mL of 0.04*M* HCl were mixed together and sonicated for 20 min. The resulting product was diluted with an equal volume of distilled water and added to a 1.0-mL plastic cuvet containing the following: 0.25 mL of BR diluted to give a final OD of between 1.0 and 2.0; 0.25 mL of 0.1*M* sodium borate, pH 9.0. A total of 0.5 mL of the diluted TMOS solution was added to the cuvet, bringing the total volume to 1.0 mL. The contents of the cuvet was thoroughly mixed and allowed to gel. Modifications of the gelled material were accomplished by allowing the sol-gel to stand at room temperature for 2–3 d. The gel was removed from the cuvet and dialyzed against the desired solution for an additional 5–7 d with several volume changes before analysis.

Sample Assay Procedures

Samples were assayed for biological activity by examination of the spectra between 370 and 700 nm. Kinetic experiments were carried out with the adduct monitored at 410 nm. All assays were performed on an HP 8452A UV/Vis Spectrophotometer with a minimum integration time of 100 μs. The light from a Kodak Carousel 35mm projector, containing an Oriel yellow filter no. 59494, which eliminated all light below 520 nm, was focused on a mirror that reflected the light through a collimating lens into the cuvet from above. The focused light was eliminated by simply placing a piece of black paper between the light source and the cuvet.

**Certain commercial equipment, instruments, and materials are identified in this article in order to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Physical Characterization of the Sol-Gel Glass

A sample of the suspension used in these studies, in 0.1*M* phosphate buffer, pH 7.0, was placed in a Coulter Submicron Particle Analyzer Model N4 MD. The sample was cooled to 20°C before analysis. The sample was analyzed for average particle size and particle size distribution. The dynamic light-scattering measurements were taken at a 90° angle from the light source.

A sample of BR sol-gel glass was prepared as previously described and further analyzed. The sample was outgassed at 100°C for 2 h before analysis by field emission electron microscopy, using a Hitachi S4100 Field Emission Scanning Electron Microscope. The dried sample was fractured, and the fractured section examined for the presence of BR membrane fragments and to visualize the macro- and micropore structure of the glassy material.

A sample of the dried BR was also examined to determine pore radius, pore volume, and pore diameter. The data were collected on a Quantachrome Autosorb-6 Automatic Volumetric Sorption Analyzer using a 40-point adsorption and a 40-point desorption test. A five-point BET surface area and total pore volume were also calculated.

Thermal Studies on the BR Containing Glass

A sample previously dialyzed at pH 7.0 was characterized for M state decay time at temperatures ranging from 0°C to 90°C. To examine further the stability of the entrapped protein, it was subjected to constant temperature at 60 and 90°C for extended time with constant monitoring. Denaturation was determined by the decrease in absorbance at 570 nm.

pH Characterization of BR Containing Glass

Sol-gel glasses with entrapped D96N BR were prepared as previously described. The gelled samples were removed from the cuvet and dialyzed against buffer at the desired pH value for 5–7 d. The buffers used were as follows: 0.1*M* sodium acetate (pH 3–6), O.1*M* sodium phosphate (pH 7,8), and 0.1*M* sodium borate (pH 9–11). Each sample was replaced in a cuvet for analysis. The effect of pH was determined for several parameters taken from the spectra and the kinetics of the M state decay. From the spectral results were calculated the maximum absorbance for each sample (A_{570max}) and the bleaching efficiency calculated as the normalized ratio of the maximum change in A_{570} nm in the light vs the ground state. This value represents the sensitivity of the BR glass as determined by the difference between the unbleached and the bleached sample divided by the maximum absorbance of the unbleached sample ($A_{570max} - A_{570bleached}/A_{570max}$). The OD at 700 nm was used for the baseline for all spectra calculations.

From kinetic data, the half-time ($T_{1/2}$) was calculated. This value was determined as half the time required to return the A_{570} nm value to the ground state after removal of illumination. The values were calculated assuming single exponential decay. The single exponential decay was chosen because all experimental data fit with less than a 2% error.

Effect of Additives on BR Glasses

The samples previously dialyzed at values of pH 5.0–11.0 were used for these studies. Each set of glasses was further dialyzed against added 10% diethanolamine (DEA) or 10% triethanolamine (TEA). These samples were dialyzed an additional 5 d before analysis. Additional samples were also subjected to dialysis against various concentrations of these amines ranging from 0.01 to 10% at pH 7.0.

After analysis of each of the samples containing 10% DEA and TEA was completed, they were subjected to the addition of increasing concentrations of guanidine, ranging in concentration from 0.001 to 1.0*M*. The pH values were readjusted and maintained for an additional week, while the samples were dialyzed with several changes of solution. These samples were similarly analyzed.

In order to ascertain whether the observed effects of the guanidine were related to the presence of the glass matrix, a series of experiments were carried out on the D96N suspension at pH 7.0 using the same concentration range of guanidine as used on the glasses. The samples were maintained at room temperature for 3 d after addition of 10% TEA and 4 d after addition of the guanidine before analysis.

RESULTS AND DISCUSSION

Preparation of the D96N-BR Containing Sol-Gel Glass

Several dilutions of buffer and water were examined in order to find the proper gelation time. If the material gelled too quickly, it was impossible to obtain good mixing. The addition of the 0.1*M* sodium borate buffer, pH 9.0, was sufficient to overcome the acidity of the TMOS preparation and still allow sufficient time for good mixing of the contents of the cuvet before gelation occurred.

Physical Characterization of the Sol-Gels

The aqueous suspension received from Pushchino was subjected to particle size analysis. The results of this analysis (Table 1) indicate that the fragments are rather uniform in size, with an average size of 554 nm. Samples assayed after several months of storage did not show any changes in particle size or particle size distribution.

Table 1
Particle Size Distribution
of D96N BR Suspension

Mean particle diameter	554 nm
Standard deviation	68 nm
Coefficient of variation	12 nm
Particle diameter distribution	
Size, nm	Distribution, %
440	26
646	74
949	0
1390	0
2040	0
3000	0

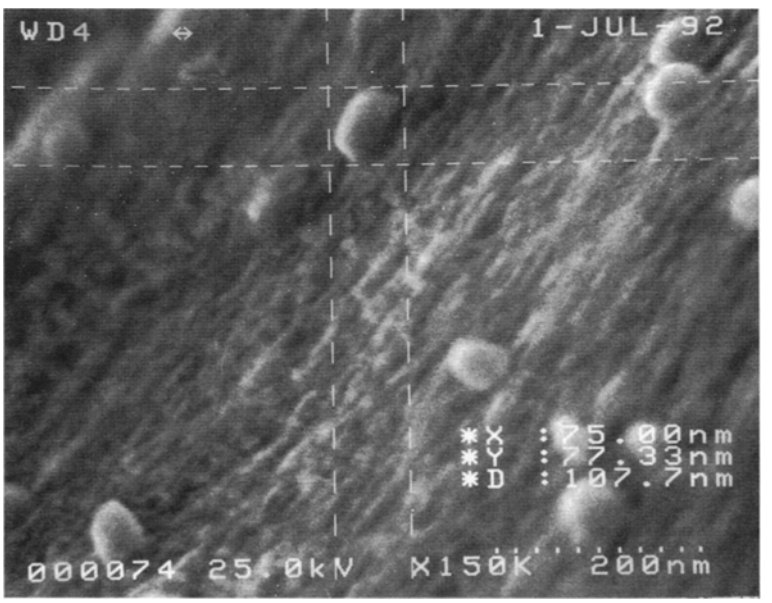

Fig. 1. Field emission scanning electron micrograph of dried D96N BR sample entrapped in sol-gel glass. The glass was outgassed at 100°C for 2 h before analysis. The particle is approx 75 × 75 × 100 nm in size.

Examination of the dried samples by field emission microscopy revealed particles that were smaller than those observed by in aqueous suspension (Fig. 1). These particles are rather oblong in shape and are approx 75–100 nm on a side. Microanalysis indicates that, for all practical purposes, the dried glass is nonporous (Table 2). Examination of sol-gel glass control samples lacking BR did not reveal the presence of these particles. These results, when compared to the dynamic light-scattering data obtained in

Table 2
Microanalysis of D96N BR
Containing Sol-Gel Glass

Average surface area	388 m^2/g
Total pore volume	0.220 cc/g
Average pore radius	11.4°A
Langmuir surface (calculated)	639 m^2/g
Correlation of calculated value with Langmuir surface data	0.999
Micropore analysis	
Micropore volume	0.136 cc/g
Micropore area	288 m^2/g
Mesopore area	98 m^2/g
Correlation to Langmuir data	0.81

Instrument was not capable of determining pores with less than 10°A radius.

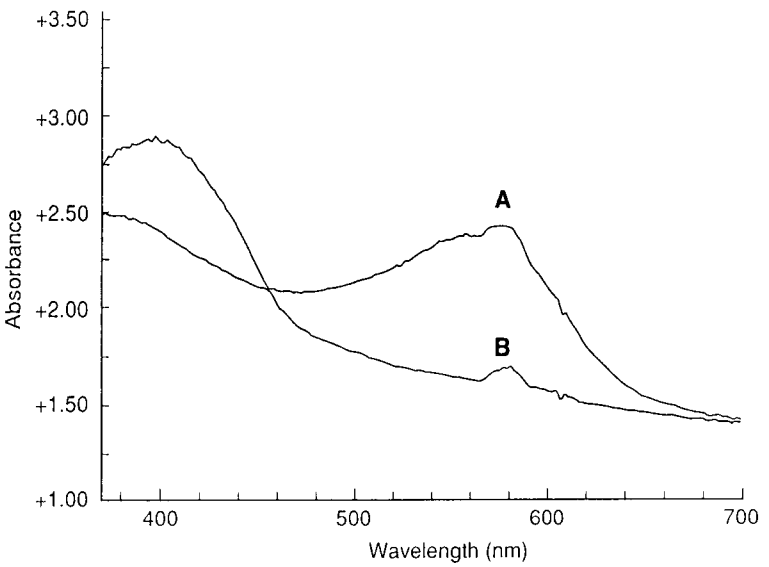

Fig. 2. Spectra taken in the ground state (A) and in light (B) of an air-dried sample of D96N BR entrapped in sol-gel glass. The glass was prepared at pH 9.0 and air-dried to a 19.9% moisture content.

aqueous suspension, indicate that on removal of water, the particles dehydrated and, as one would expect, were greatly decreased in size. This dehydration process does decrease the activity of the protein. However, activity is still reasonable with limited water content. Figure 2 shows the spectra of an air-dried sample previously dialysed at pH 9.0. This sample showed a $T_{1/2}$ value of 8 s for M state decay (Fig. 3), which is identical to the "wet" sample in the sol-gel. The bleaching efficiency was

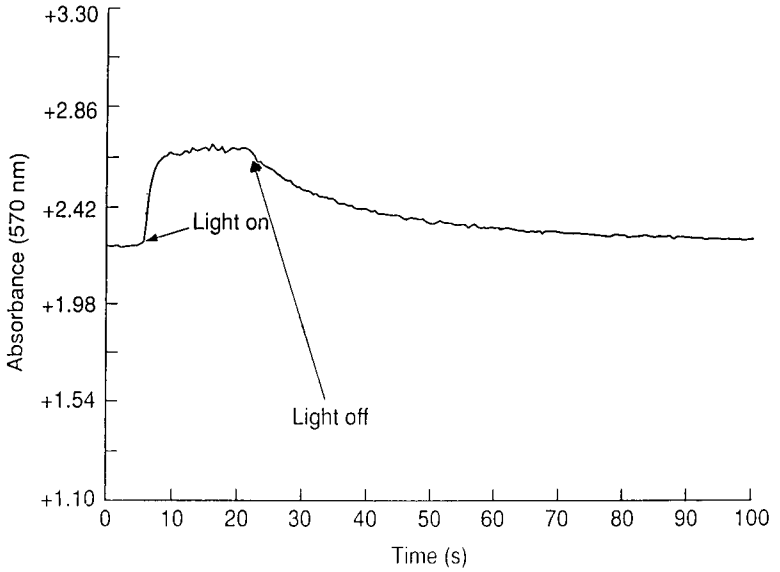

Fig. 3. Kinetic response of the same sample shown in Fig. 2. Monitored at 410 nm using a light source with a filter blocking all light below 520 nm.

approx 0.66 for the air-dried sample, whereas the "wet" sample had an efficiency of >0.90. Therefore, it does appear that activity decreases with decreasing water content. On heating overnight at 100°C in a vacuum oven, the sample lost 19.9% additional weight. Previous studies with wild-type BR have shown that when immobilized in an organic polymer dried to 20% water content, it had approx 10 times the M lifetime than the aqueous suspension (3). This phenomenon was not observed with the glass-entrapped D96N mutant.

pH Characterization

Sol-gel samples dialyzed at the desired pH for 1 wk were analyzed as previously described. Figure 4 shows the maximum absorbance values vs pH for a series of dialyzed D96N control and amine-containing gels. These data show a strong maximum at pH 6.0–7.0 for entrapped samples. Similar results were observed with the BR suspension. Previously published data on suspended material (7) indicate that over a pH range of 4–8, the time constant for M state decay, photovoltage, and H^+ pumping of the D96N mutant increases exponentially with increasing pH. Our studies on $T_{1/2}$ values vs pH also show similar results for both control and amine-containing samples (Fig. 5). The bleaching efficiency of the entrapped mutant BR (Fig. 6)follows a similar trend between pH 4 and 8 as described for the suspension (7). Between pH 8 and 11, the efficiency remains constant at >90%. Although the efficiency is >90%, the quantity of active BR decreases drastically between the peak at pH 6 and 11 (Fig. 4). The data, as

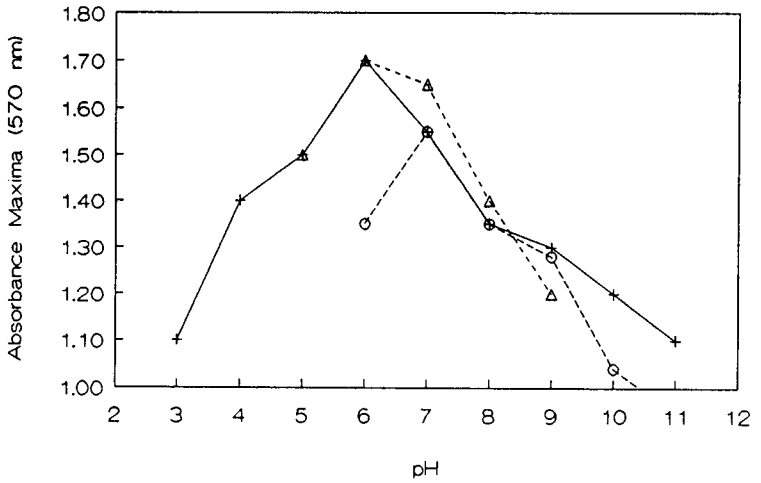

Fig. 4. Absorbance maxima $A_{570\ nm\ max}$ vs pH for D96N BR entrapped in sol-gel glasses. Calculated from spectra at each pH value taken in the ground state. The effect of 10% DEA and 10% TEA on the absorbance maxima ($A_{570\ nm}$) vs pH as compared to a control sample with no additives. X represents the control samples, triangles represent the 10% DEA samples, and O represent the 10% TEA samples.

expected, show that the M state decay times of the entrapped BR are directly related to the external pH values. This has been well documented for D96N purple membrane material (*7,27,31–33*).

Thermal Studies

A sample of entrapped D96N BR mutant previously adjusted to pH 7.0 was sequentially subjected to increasing temperatures from 0 to 90°C. The $T_{1/2}$ values were determined at 10°C intervals after removal of the light source from the sample. The data were plotted against the temperature in an Arrhenius-type plot (Fig. 7). Groma and Dancshazy (*34*) examined the time constants for wildtype BR using one, two, and three exponential fits to the M state decay times. Although they found that the three exponential decay model fits best, all three models yielded a temperature dependence Arrhenius-type curve with Rval of >0.93. Using the value of the single exponential decay model, the date (Fig. 5) showed an Rval of 0.997 vs theoretical. Therefore, as expected, the immobilized BR follows the Arrhenius Law.

It was of interest to note that when the BR was entrapped in a polyacrylamide gel (*34*), it showed modified decay kinetics, losing one of the three exponential components. It would be of interest to determine whether our material showed similar results. Future studies will include a more detailed analysis of the kinetic characteristics of the immobilized BR.

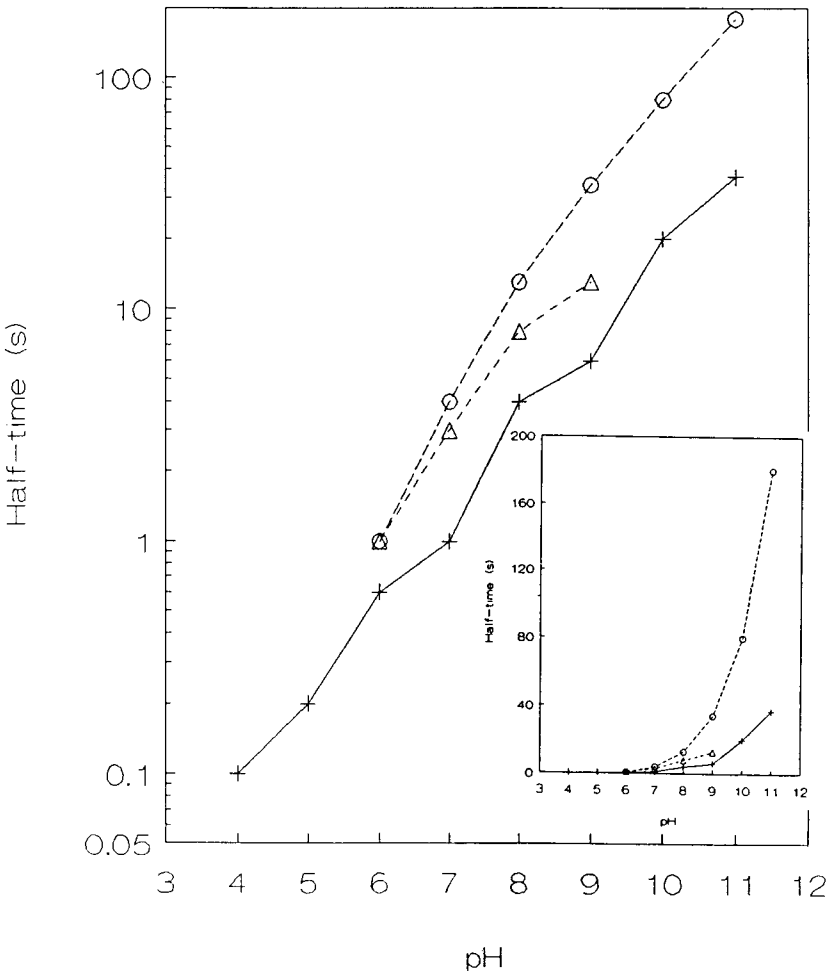

Fig. 5. Half-time value ($T_{1/2}$) for 10% DEA- and 10% TEA-treated D96N Br entrapped in sol-gel glass as compared to a control that has not been treated with amine. X represents the control samples, O represent the 10% TEA samples, triangles represent the 10% DEA samples. The data shown in the insert are presented in linear form to show better the difference between the control and the TEA samples.

Because we are interested in the long-term stability of the entrapped BR, it was also of interest to estimate the thermal stability at high temperature by monitoring the entrapped BR at several temperatures for extended time periods. Samples were held at 60 and 90°C for periods of up to 2 h. The change in A_{570nm} was monitored vs time. Results indicate that the entrapped proteins loose half their activity in 8.3 h at 60°C and 18.3 min at 90°C. The BR suspension showed similar results.

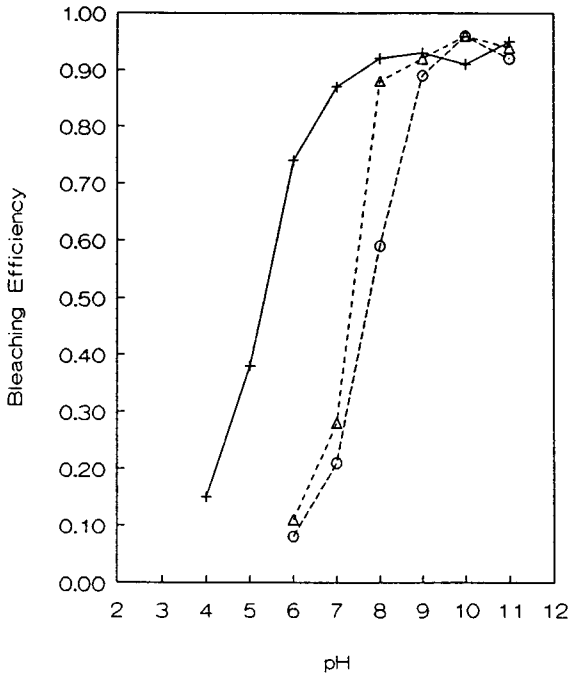

Fig. 6. Bleaching efficiency of the D96N BR entrapped in sol-gel glass vs pH. The data were plotted as the difference between the absorbance maxima at 570 nm in the ground state minus the adsorbance at 570 nm in the light divided by the 570-nm absorbance maxima. X represents the control, triangle represents 10% DEA samples, and O represents 10% TEA samples.

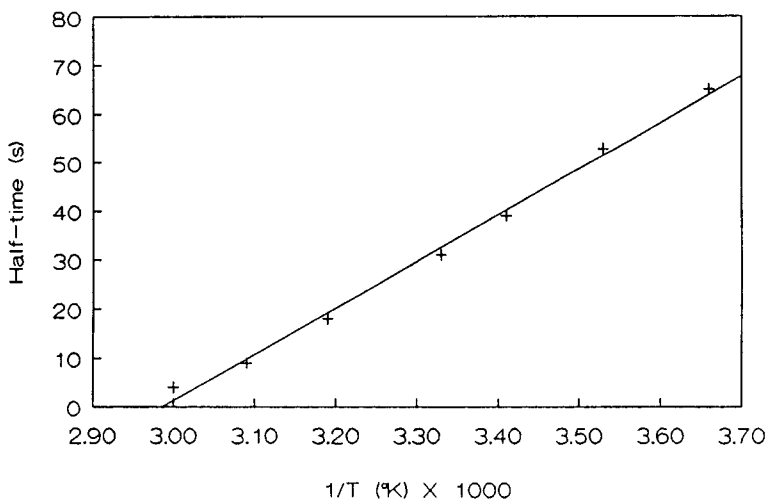

Fig. 7. Arrhenius-type plot of the half-time values ($T_{1/2}$) vs the inverse of temperature (°K). The calculated Rval for the theoretical straight line is 0.997.

Effect of Additives

Either 10% DEA or 10% TEA was added to samples of entrapped BR at pH 5–11. These samples, in the pH-adjusted amine solutions, were dialyzed for 1 wk before analysis. The A_{570max} values for the DEA- and TEA-treated samples were similar to the data presented for the untreated samples at the same pH values (Fig. 4). The TEA-treated samples did reveal an A_{570max} at pH 7. In addition, the TEA-treated samples showed lower absorbance maxima as compared to the untreated samples at the same pH values. The bleaching efficiency of the treated samples was similar to the untreated samples (Fig. 6) over the same pH range, exceeding 90% above pH 8. The major differences were observed in the $T_{1/2}$ values for the amine-containing samples (Fig. 5). All treated samples showed increased M state decay times with the greatest differences observed with the TEA-treated samples, where the maximum $T_{1/2}$ values were extended by five-fold over the control at the highest pH values examined.

Samples at pH 7, when exposed to increasing concentrations of TEA ranging from 0.1 to 10%, showed $T_{1/2}$ values between <1 and 12 s, indicating that there is a relationship between amine concentration and M state decay time.

The addition of guanidine to the amine-treated samples was expected to act in a manner similar to the amines by decreasing the M state decay rates, as previously observed in the wild-type BR (*28–30,35*). Surprisingly, the opposite occurred. Samples of TEA- and DEA-treated entrapped BR were exposed to 0.4*M* guanidine and maintained at the appropriate pH for 5 d before analysis. It was found that only minor changes had occurred in the A_{570max} vs pH. However, large differences were observed in the bleaching efficiency (Fig. 8). In the case of the guanidine-treated samples, the bleaching efficiency decreased markedly between pH 6 and 8 such that the resulting values resembled that of the wild-type rather than the D96N mutant. This was confirmed by comparing the $T_{1/2}$ values for the guanidine-treated samples with the untreated DEA and TEA samples (Fig. 9). At the higher pH values, the $T_{1/2}$ was considerably decreased when the amine was replaced by the 0.4*M* guanidine. To determine the extent of this effect, a series of experiments were carried out with 10% TEA, pH 7, treated samples, which were further dialyzed against several changes of guanidine at concentrations from 0.001 to 1.0*M* maintained at pH 7. Results indicated that all samples had decreased $T_{1/2}$ values of <1 s. A series of experiments with the D96N suspension at pH 7 yielded similar results. All samples showed $T_{1/2}$ values of <1 s. Variations in the glass-entrapped BR bleaching efficiency, however, did occur, ranging from a low of 0.15 (15%) at 1.0*M* guanidine to a maximum of 0.88 (88%) in 0.001*M* guanidine (Fig. 10). The A_{570max} values of the entrapped BR were similar to the control sample, except for the 4.0*M* guanidine samples, which were approx 60% of the control.

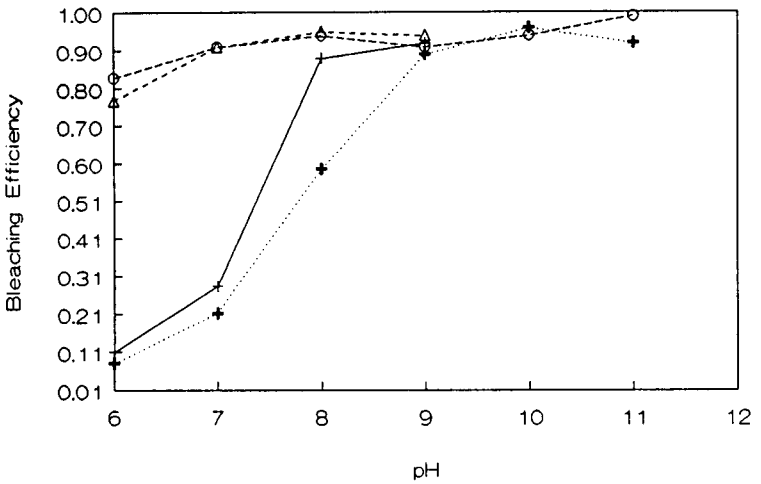

Fig. 8. Bleaching efficiencies of D96N BR entrapped in sol-gel glass treated with 10% DEA and 10% TEA vs increasing pH values with and without the addition of 0.4*M* guanidine. Triangles represent the 10% DEA samples, X represents the 10% TEA samples, O represents 10% TEA samples treated with 0.4*M* guanidine, and + represents 10% DEA samples treated with 0.4*M* guanidine. For a description of bleaching efficiency, *see* the legend for Fig. 6.

These data indicate first that the observed changes caused by the introduction of amines or guanidine are reversible. Dialysis of one compound from the glass and introduction of another confer the effects of the newly introduced additive agent and eliminate the effects of the previous agent.

The addition of 0.4*M* guanidine to a sample at pH 7 containing 10% TEA, while maintaining the TEA concentration, caused an increase in the M state decay rate similar to that observed after dialysis of the sample to remove the TEA and add guanidine. However, at the lowest concentration examined, 0.001*M* guanidine, only a very minor difference was observed. The guanidine concentration required in the presence of the 10% TEA was greater in order to accomplish the same degree of change in the BR kinetics.

It has already been reported that sodium azide at increasing concentration (32) acts in a manner similar to H^+ concentration in the D96N mutant. It also appears that guanidine has the same effect as sodium azide on the M state decay rate. The guanidine may act as a proton carrier therefore increasing the reprotonation rate. Otto et al. (32) found that azide increases the M decay rate proportionally to the azide concentration and can become >100 times greater than in wild type. The authors claimed that the azide speeds up the slow charge translocation. The M decay remains essentially monophasic in the presence of azide and exerts it effect on D96N by a dynamic association-dissociation equilibrium that is faster than the observed M decay. The guanidine data appear similar. Guanidine increases the M decay rate proportionally to concentration and

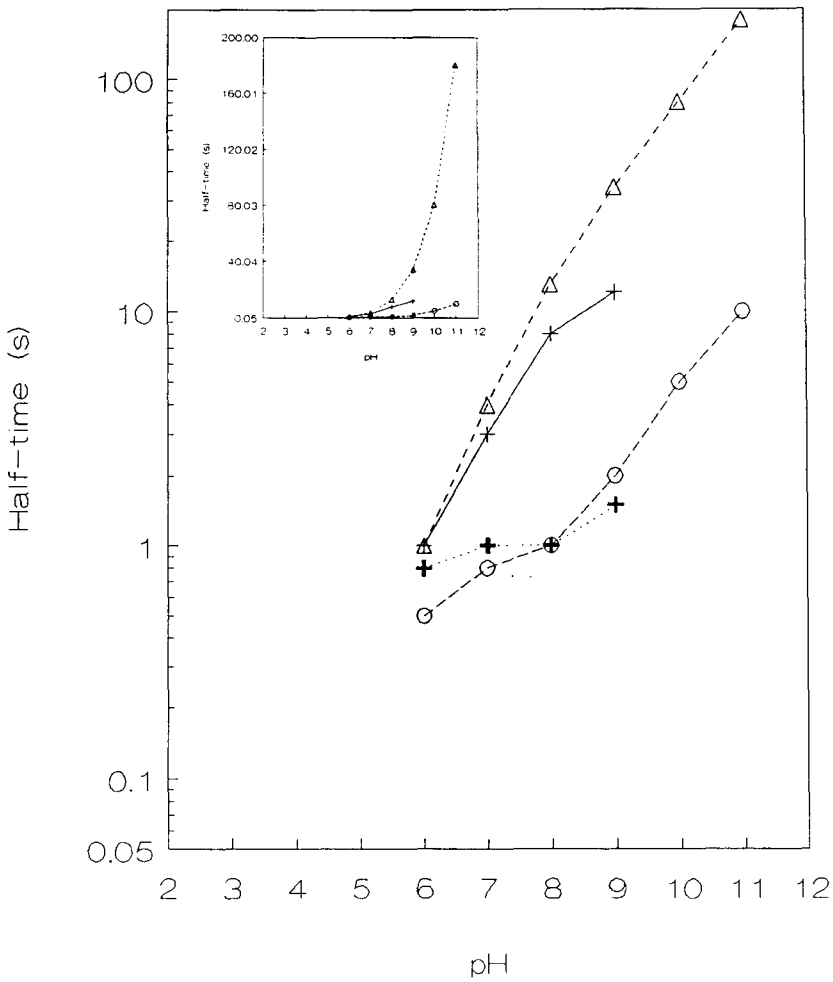

Fig. 9. Half-time values ($T_{1/2}$) in seconds (s) for entrapped D96N BR vs pH for amine-treated samples before and after treatment with 0.4*M* guanidine. Insert: X represents the 10% DEA samples before treatment with guanidine, triangles represent 10% TEA samples before treatment with guanidine, O represent 10% TEA samples after treatment with guanidine, and + represents DEA samples after treatment with guanidine. Insert represents the data in linearized form.

causes the entrapped D96N mutant to act more like the wild-type than the untreated mutant control. The effect is pH-dependent. All of these characteristics have been described for azide (32). Whether this effect is the result of binding of the guanidine to the Asn96 is impossible to state at this time. Since the only difference between the wild-type and the D96N mutant is the Asp96 substitution, it is logical to assume that some kind of molecular association has occurred at this location in the BR molecule. This association then causes the kinetic modification.

This article describes some of the characteristics of the D96N mutant BR when immobilized in sol-gel glass. Since we know it is now possible to dry samples to 20% water content, future plans will include a detailed

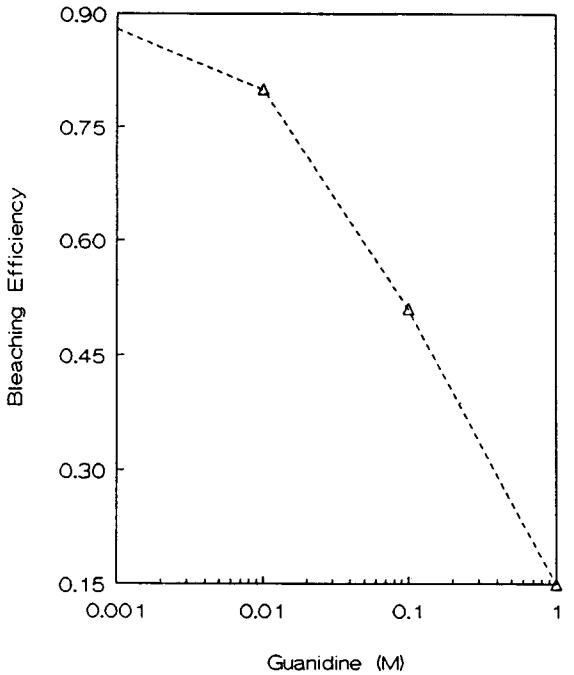

Fig. 10. Bleaching efficiency at pH 7.0 of D96N-entrapped samples treated with 0.001–4.0*M* guanidine.

study of the drying process and methods for the maintenance of biological activity during drying. In addition, we will continue to develop new methods for decreasing the M state decay rate.

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