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Chemiluminescence of the Mn^{2+}-Activated Ribulose-1,5-bisphosphate Oxygenase Reaction: Evidence for Singlet Oxygen Production†

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ABSTRACT: Chemiluminescence has been observed during catalysis by Mn^{2+}-activated ribulose-bisphosphate carboxylase/oxygenase from spinach. The luminescence is ribulose 1,5-bisphosphate (RuBP) and O_2-dependent and is inhibited by 2-carboxyarabinitol 1,5-bisphosphate and high concentrations of bicarbonate; it is therefore ascribed to the RuBP oxygenase activity. The luminescence is inhibited by azide and enhanced in D_2O and in the presence of diazabicyclooctane. The emission maximum is between 620 and 660 nm. The initial rate of light emission is second order in enzyme concentration. The data strongly suggest that singlet oxygen is produced during turnover, that the observed chemiluminescence is due to dimol emission of singlet oxygen, and that this provides a basis for a highly sensitive assay for RuBP oxygenase.

The enzyme D-ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO),[1] as the name implies, catalyzes both the carboxylation and oxygenation of RuBP. These reactions are the initial steps in photosynthesis and photorespiration, respectively [for a review, see Miziorko and Lorimer (1983)]. Although the oxygenase activity competes for RuBP with the carboxylase activity and the resultant photorespiration appears to oppose photosynthesis, all RuBP carboxylases studied to date catalyze oxygenation. It has been proposed that the

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[1] Abbreviations: CABP, 2-carboxyarabinitol 1,5-bisphosphate; cps, counts per second; DABCO, diazabicyclooctane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; $^1O_2$, singlet oxygen; $^3O_2$, triplet oxygen; RuBisCO, D-ribulose-1,5-bisphosphate carboxylase/oxygenase; RuBP, D-ribulose 1,5-bisphosphate; SOD, superoxide dismutase.

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oxygenase activity is an inherent consequence of the chemistry of the carboxylase reaction (Lorimer, 1973). However, little information regarding the oxygenase reaction is available.

In higher plants and cyanobacteria, the enzyme is hexadecameric, consisting of eight 53-kDa large (L) and eight 15-kDa small (S) subunits. The active site is located on the large subunit, while the function of the small subunit remains unknown [for a review, see McFadden et al. (1986)]. In the photosynthetic bacterium *Rhodospirillum rubrum*, the enzyme is a homodimer consisting of two large subunits (Tabita & McFadden, 1974).

Catalysis by either carboxylase or oxygenase requires activation of the enzyme by CO_2 and a divalent metal cation (Miziorko & Lorimer, 1983). Although Mg^{2+} results in the highest carboxylase:oxygenase ratio, and is believed to be the activating metal ion in vivo, other metal ions can also contribute to activation of the enzyme, resulting in various carboxylase:oxygenase ratios (Christeller, 1981). This ratio is often expressed in the form of the specificity factor, defined as V_cK_O/V_OK_c, where V_c and V_O are the maximal velocities and K_c and K_O are the Michaelis constants for CO_2 and O_2, respectively (Laing et al., 1974; Jordan & Ogren, 1983). For example, when Mn^{2+} is used in place of Mg^{2+}, the specificity factor for the spinach enzyme decreases 25-fold. This is due mainly to a decrease in K_O. The specificity factor changes similarly for RuBisCO from other higher plants, a green alga, a cyanobacterium, and *R. rubrum* (Jordan & Ogren, 1983). There are differences in the way in which the two metal ions participate in the oxygenase mechanism as well (Kreckl et al., 1989). We now report evidence which indicates that when RuBisCO is activated with Mn^{2+}, singlet oxygen is produced as a result of the oxygenase reaction, resulting in chemiluminescence that can be conveniently detected with a liquid scintillation counter.

Experimental Procedures

Materials. SOD and the sodium salts of RuBP and azide were obtained from Sigma Chemical Co. DABCO was purchased from Aldrich. D_2O was purchased from KOR Isotopes. CABP was synthesized as described by Pierce et al. (1980). RuBisCO was purified from spinach as described by Berhow et al. (1982).

Chemiluminescence Measurements. A Beckman LS-9000 liquid scintillation counter was used to measure light emission. Unless otherwise indicated, the reactions were initiated by adding the sample (1.5 mL) to 200 μL of 10 mM RuBP equilibrated to 25 °C in a 3-mL fluorescence cuvette that had been placed inside a 20-mL glass liquid scintillation vial. Counts were recorded manually every 10 s starting from 10 s after initiation; orientation of the cuvette within the vial did not affect the count rate. Luminescence rates (cps) were determined from the slope of the line obtained by plotting counts vs time. Before being mixed with RuBP, samples were incubated at 25 °C for 30 min except where noted. Unless otherwise specified, they contained 50 mM MOPS (pH 7.5), 1 mM $MnCl_2$, 1 mM $NaHCO_3$, and RuBisCO (0.1 mg/mL, 2 units of RuBP carboxylase/mg). The use of plastic gloves in manipulating vials was avoided because such handling created static electricity that resulted in false counts.

Measurements of Oxygen Consumption. Oxygen concentrations and oxygen consumption was measured by using a Hansatech oxygen electrode and a Rec-482 Pharmacia chart recorder. Various oxygen concentrations were obtained by mixing various amounts of 50 mM MOPS (pH 7.5) bubbled with O_2 or Ar. The O_2 concentrations were measured immediately before and continuously throughout the reaction.

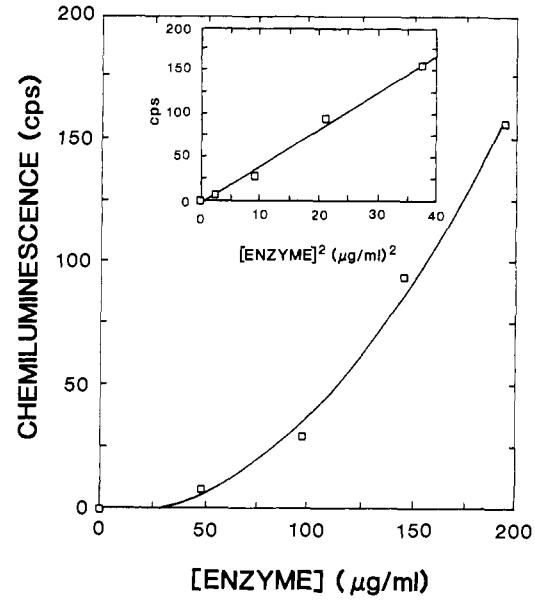


FIGURE 1: Chemiluminescence as a function of protein concentration. The inset shows the chemiluminescence as a function of the protein concentration squared.

Spectral Distribution. The following Wratten gelatin filters (Kodak) were used. Shown in parentheses are the wavelengths (in nm) at which the filters have an 80% transmission: 2A (435), 8 (515), 12 (535), 22 (580), 25 (620), 92 (660), and 70 (700). All filters were cut to 7.8 by 4.0 cm such that when rolled and placed inside the 20-mL glass scintillation vial, they would expand to line the wall. The rates of light emission with and without the filters were obtained. Relative light emission between two wavelengths was determined from the decrease in emission rate due to a particular filter. Wavelengths that correspond to 80% transmission, although partly arbitrary, were chosen since to choose a point at lower transmission would be to ignore a part of the spectrum where a filter would absorb significantly. For full spectral information on these filters, see Kodak publication B-3, Kodak Filters for Scientific and Technical Uses. Counts were corrected for the sensitivity of the photomultiplier tube by averaging the sensitivities within the wavelength interval between a given pair of filters. Since the sensitivity declines rapidly between 600 and 700 nm (Horrocks, 1974), the corrections are approximate.

Results

Protein Dependence. When the rate of light emission was plotted as a function of protein concentration, it was found that the rate was second order in protein concentration (Figure 1). This is consistent with the source of the luminescence being the dimol emission of singlet oxygen (see Discussion).

Mn^{2+} Dependence. Chemiluminescence was dependent on added Mn^{2+} in an apparent Michaelis–Menten manner (Figure 2). The $K_m^{Mn^{2+}}$ was 0.30 mM. In addition, the following metal ions were tested: Mg^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+}, Ni^{+2}, Cu^{2+}, and Co^{2+}. None other than Mn^{2+} supported luminescence (data not shown). Magnesium ion was found to inhibit luminescence, probably by competing with Mn^{2+} for the activation sites (Figure 2, inset).

RuBP Oxygenase Activity and Chemiluminescence. As shown in Figure 3, chemiluminescence shows normal saturation by RuBP. K_m^{RuBP} was 1.4 μM. However, these results must be interpreted with caution. As is the case with the protein concentration dependence (mentioned above), the observed luminescence may actually be linearly dependent on $[RuBP]^2$ at very low [RuBP] (inset, Figure 3).

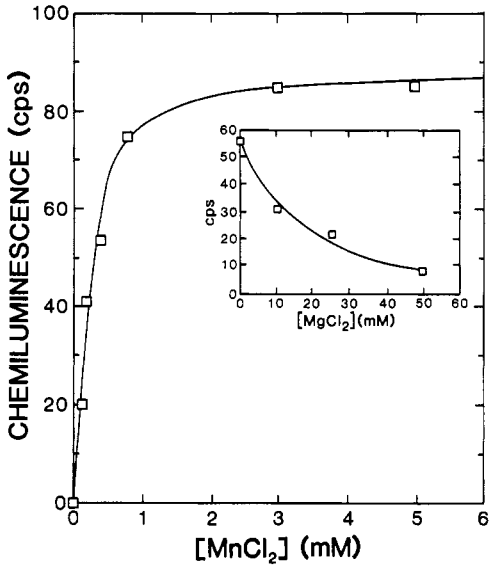


FIGURE 2: Chemiluminescence as a function of $[Mn^{2+}]$ after activation of the enzyme for 60 min. The effect of Mg^{2+} on the chemiluminescence in the presence of 1 mM $MnCl_2$ is shown in the inset.

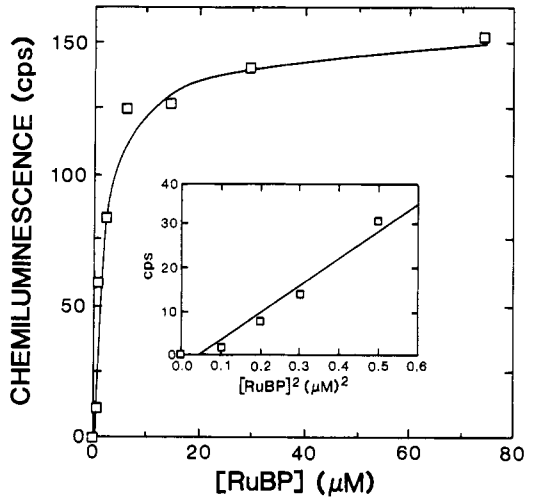


FIGURE 3: RuBP dependence of the chemiluminescence. The inset shows the dependence of chemiluminescence upon $[RuBP]^2$ in the concentration range of 0.1–0.5 μM RuBP. The data shown in the inset were obtained by using a sample volume of 3.0 mL to which RuBP was added and with enzyme that had been activated for 60 min.

Table I: Effect of Various Substances on Chemiluminescence[a]

compound	% emission rate	compound	% emission rate
control	(100)	azide	4
DABCO	315	SOD	100
ascorbate	35	CABP	0

[a] Concentrations used were DABCO, 0.5 mM; ascorbate, 5 mM; azide, 1 mM; SOD, 180 units/mL; and CABP, 25 μM.

The luminescence required low concentrations of bicarbonate but was inhibited by high concentrations (Figure 4). The oxygen dependence of the luminescence is shown in Figure 5. Also shown is the oxygen dependence of oxygen consumption. As can be seen, the two observables saturate simultaneously and appear to correlate over the range of oxygen concentrations used. Additionally, the luminescence was abolished in the presence of 25 μM CABP, as would be expected for the RuBP oxygenase reaction.

Singlet Oxygen. Azide, a known quencher of singlet oxygen (Foote et al., 1972), abolished light emission almost completely (Table I). DABCO, also a known $^1O_2$ quencher (Ogryzlo & Tang, 1970), stimulated the emission rate approximately 3-fold. Superoxide dismutase had no effect on the intensity or rate of light emission, indicating that free superoxide ion was not necessary for chemiluminescence.

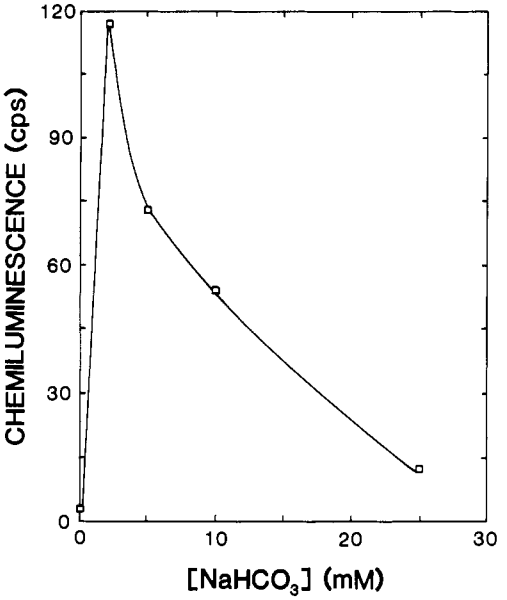


FIGURE 4: Chemiluminescence as a function of bicarbonate concentration. The MOPS buffer (see Experimental Procedures) was bubbled with O_2 gas for 2 h prior to the addition of protein, $NaHCO_3$, and $MnCl_2$.

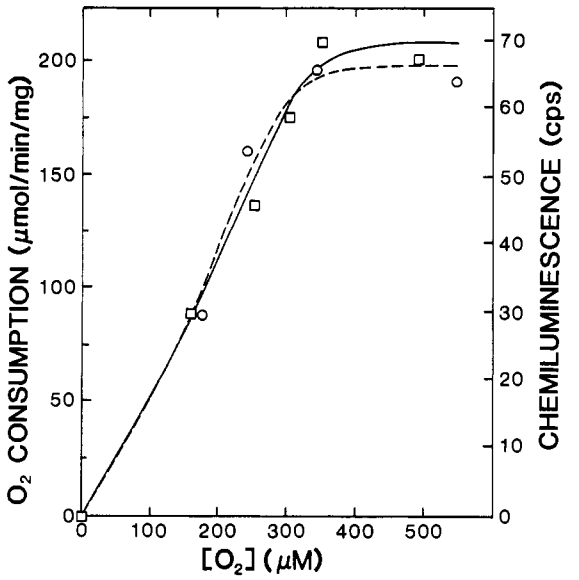


FIGURE 5: The solid line represents chemiluminescence (□) and the dashed line represents oxygen consumption (○) as a function of O_2 concentration. Other components of the incubation mixtures were as described in the text. The protein concentration used was 0.9 mg/mL.

Singlet oxygen is known to have a longer lifetime in D_2O than in H_2O (Kajiwara & Kearns, 1973). As shown in Figure 6, the luminescence due to RuBP oxygenase was greatly enhanced in D_2O. The rate for the oxygenase activity, as measured with the O_2 electrode, was not enhanced in D_2O (data not shown).

Due to the weakness of the emitted light, all attempts to measure a continuous spectrum were unsuccessful. A discontinuous spectrum, corrected as described under Experimental Procedures for the wavelength dependence of the sensitivity of our photomultiplier tube, was determined. The percentages of detected light were 6%, 56%, and 38% between

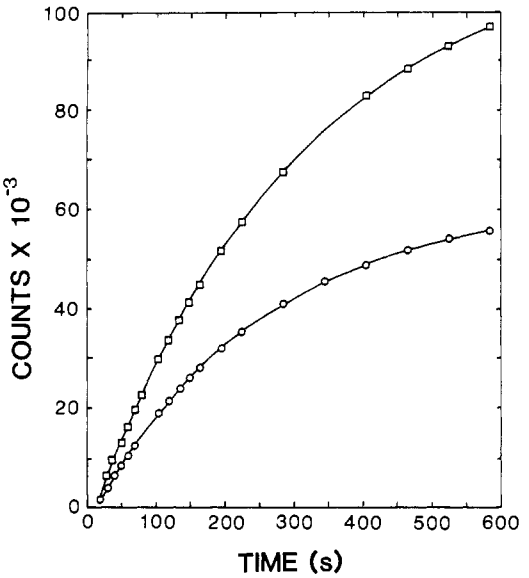


FIGURE 6: Chemiluminescence in the MOPS–Mn^{2+}–bicarbonate buffer containing H_2O (○) and 50% D_2O (□).

wavelength ranges of 580–620, 620–660, and 660–700 nm, respectively. Light above 700 nm was not detected, possibly due to the rapid decline in sensitivity of the photomultiplier tube in this spectral region.

Discussion

The observed chemiluminescence displays saturation kinetics with respect to Mn^{2+}, RuBP, and O_2. Bicarbonate (CO_2) is required in low concentrations but the chemiluminescence is inhibited at higher concentrations. The emission is abolished in the presence of CABP. Collectively these observations establish that the chemiluminescence is a result of the oxygenase activity of RuBisCO (Miziorko & Lorimer, 1983).

The inhibition of the luminescence by azide, the enhancement of it in D_2O, and the lack of inhibition by superoxide dismutase are all consistent with $^1O_2$ being the source of light emission (Spikes & Swartz, 1978).

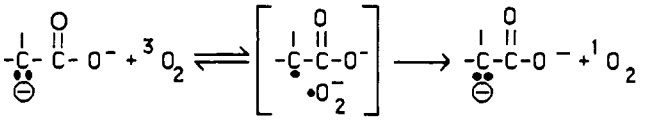
A unique feature of molecular oxygen is that it exists in a triplet ground state (designated $^3\Sigma_g^-$) in which the outer two electrons remain unpaired and have parallel spins. This accounts for the paramagnetic and diradical-like properties of O_2. The lowest excited electronic state is a singlet state designated $^1\Delta_g$. Many systems that produce $^1O_2$ result in chemiluminescence. Quite often, especially in the gas phase, this is not simply due to the decay of individual O_2 molecules in the $^1\Delta_g$ state to the $^3\Sigma_g^-$ state, but rather a simultaneous decay of two O_2 molecules in the $^1\Delta_g$ state to the ground $^3\Delta_g$ state. Although no O_2 dimer is ever formed, the chemiluminescence due to this process is often termed dimol emission (Wilson & Hastings, 1970). The emission maximum for this process occurs at 634 nm (Khan & Kasha, 1970), and our spectrum suggests that this is occurring in the presently described system.

The enhancement of the luminescence by DABCO also suggests that it is the dimol emission that produces the light. Although DABCO is a known quencher of singlet oxygen and inhibits emission due to single-molecule transitions, it is known to stimulate emission due to dimol emission (Ogryzlo & Tang, 1970; Deneke & Krinsky, 1976). Further supporting dimol emission as the source of chemiluminescence is the observation that the initial rate of light emission is second order in enzyme concentration. A similar observation has been made in the well-studied hypochlorite reaction:

$$H_2O_2 + ClO^- \rightarrow {}^1O_2 + Cl^- + H_2O$$

Chemiluminescence from this reaction is due to dimol emission. As this emission is second order in $[^1O_2]$, it is also second order in $[OCl^-]$ (Deneke & Krinsky, 1977). Thus, if the luminescence of the RuBP oxygenase reaction is due to dimol emission of $^1O_2$, then it might be expected that the emission rate would be second order in enzyme concentration and perhaps in substrate and metal ion concentrations as well.

The mechanism by which singlet oxygen is formed is unknown. However, it is known that $^1O_2$ can be produced when molecular oxygen reacts with α-carboxyl carbanions (Adam et al., 1982):



The reaction proceeds by a single electron transfer from the carbanion to $^3O_2$, resulting in a superoxide ion–enolate radical cage. The electron can then be transferred back to the enolate, regenerating the carbanion and either triplet or singlet oxygen. It has been proposed that the RuBP oxygenase reaction goes through a very similar mechanism, the superoxide ion–enolate radical cage being an intermediate (Lorimer, 1981). Thus it is conceivable that $^1O_2$ is produced by the carbanion form of the RuBP enolate.

It is tempting to speculate that the capacity of Mn^{2+} to participate in one electron transfer reactions may account for its support of chemiluminescence. However, other metals tried, such as Co^{2+} and Ni^{2+}, which may also participate in one-electron transfers as well as the oxygenase reaction (Wildner & Henkel, 1979; Christeller, 1981), did not support chemiluminescence. One possibility is that bound Mn^{2+} may directly or indirectly result in stabilization of the $RuBP^-$ carbanion, allowing $^1O_2$ to be produced. Whatever the mechanism may be, it is clear that there is something fundamentally different for the Mn^{2+}-dependent RuBP oxygenase reaction that results in chemiluminescence.

Currently, the most convenient method for measuring RuBP oxygenase activity is by the use of the O_2 electrode. However, the turnover number of the enzyme is low (McFadden, 1973; Miziorko & Lorimer, 1983), as is the sensitivity of the electrode. Consequently, large amounts of protein are required. Moreover, an oxygen electrode is not always available. As can be seen in Figure 5, the ratio of oxygenase activity to the rate of light emission is relatively constant over the range of oxygen concentrations used. In our hands, chemiluminescence appears to afford approximately a 70-fold more sensitive assay and only requires access to a liquid scintillation counter. For example, 0.01 unit of RuBP oxygenase was sufficient for the chemiluminescent assay whereas ca. 0.7 unit was necessary for detection by our O_2 electrode. Of course, each system must be calibrated initially with an oxygen electrode. Although the chemiluminescence is only observed with the Mn^{2+} form of the enzyme, the assay can be performed in the presence of Mg^{2+}, if its presence is taken into account (Figure 2). The second-order dependency on enzyme concentration must also be considered in the chemiluminescent assay for RuBP oxygenase.

It is generally accepted that the Mg^{2+} form of RuBisCO is the catalytically active species in vivo. However, considering that the K_m for O_2 of the higher plant enzyme in the presence of Mn^{2+} is 20-fold lower than in the presence of Mg^{2+} (Christeller, 1981), and considering the potential harm of in vivo production of singlet oxygen by an enzyme as abundant

as RuBisCO, quantification of Mn^{2+}-activated RuBisCO in chloroplasts will be important.

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A Delay in Membrane Fusion: Lag Times Observed by Fluorescence Microscopy of Individual Fusion Events Induced by an Electric Field Pulse

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ABSTRACT: Low light level video microscopy of the fusion of DiI- (1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) labeled rabbit erythrocyte ghosts with unlabeled rabbit erythrocyte ghosts, held in stable apposition by dielectrophoresis in sodium phosphate buffers, showed reproducible time intervals (delays) between the application of a single fusogenic electric pulse and the earliest detection of fluorescence in the unlabeled adjacent membranes. The delay increased over the range 0.3–4 s with a decrease in (i) the electric field strength of the fusion-inducing pulse from 1000 to 250 V/mm, (ii) the decay half-time of the fusogenic pulse in the range 1.8–0.073 ms, and (iii) the dielectrophoretic force which brings the membranes into close apposition. A change in the buffer viscosity from 1.8 to 10 mP·s caused the delay to increase from 0.36 to 3.7 s (in glycerol solutions) or to 5.2 s (in sucrose solutions). The delay decreased 2–3 times with an increase in temperature from 21 to 37 °C. It did not differ significantly for "white" ghosts [0.013 mM hemoglobin (Hb)] or "red" ghosts (0.15 mM Hb) or buffer strength over the range 5–60 mM (sodium phosphate, pH 8.5). The calculated activation energy, 17 kcal/mol, does not depend on the field strength. The yield of fused cells was high when the delay was short. The delay in electrofusion resembles the delays in pH-dependent fusion of vesicular stomatitis viruses with erythrocyte ghosts [Clague, M. J., Schoch, C., Zech, L., & Blumenthal, R. (1990) *Biochemistry 29*, 1303–1308] and of fibroblasts expressing influenza hemagglutinin and red blood cells [Morris, S. J., Sarkar, D. P., White, J. M., & Blumenthal, R. (1989) *J. Biol. Chem. 264*, 3972–3978]. The delay reflects the lifetime of a long-lived intermediate state in fusion. It may be partly due to the time of mutual approach of membranes needed to reach molecular contact and/or the time of relatively slow molecular rearrangements leading to formation of intermembrane connections.

Membrane fusion is a process which converts two membranes into one, thereby permitting membrane mixing and the removal of the membrane barrier or contents mixing. One proposal, for example, suggests that cell fusion involves six possible stages (Rand & Parsegian, 1986): (i) stable membrane apposition, (ii) triggering of fusion, (iii) contact, (iv) focused destabilization, (v) membrane coalescence, and (vi) restabilization. The time interval between the triggering and the membrane coalescence (the actual fusion event) is usually considered to be very short. Time-resolved freeze–fracture electron microscopy suggests that during neurotransmitter release this time (delay in fusion) is less than 5 ms (Heuser et al., 1979). This is the same order of magnitude of the time for exocytosis in other systems (Plattner, 1989). Studies of

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