Kojima, N., Fox, J. A., Hausinger, R. P., Daniels, L., Orme-Johnson, W. H., & Walsh, C. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 378.
Laemmli, U. K. (1970) *Nature (London) 227*, 680.
Lindahl, P. A., Kojima, J., Hausinger, R. P., Fox, J. A., Teo, B. K., Walsh, C. T., & Orme-Johnson, W. H. (1984) *J. Am. Chem. Soc. 106*, 3062.
Livingston, D. J., Fox, J. A., Orme-Johnson, W. H., & Walsh, C. T. (1987) *Biochemistry 26*, 4228.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265.
Maniatis, T., Fritsch, E. F., & Sambrook, J., Eds. (1986) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Matsubara, H., Inoue, K., Hase, T., Hiura, H., Kakuno, T., Yamashita, J., & Horio, T. (1983) *J. Biochem. (Tokyo) 93*, 1385.
Mills, D., & Kramer, F. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2232.
Moura, I., Moura, J. J. G., Santos, H., Xavier, A. V., Burch, G., Peck, H. D., Jr., & LeGall, J. (1983) *Biochim. Biophys. Acta 742*, 84.
Muth, E., Morschel, E., & Klein, A. (1987) *Eur. J. Biochem. 169*, 571.
Ohtsuka, E., Matsuki, S., Ikehara, M., Takahashi, Y., & Matsubara, K. (1985) *J. Biol. Chem. 260*, 2605.
Reeve, J. N., Beckler, G. S., Cram, D. S., Hamilton, P. T., Brown, J. W., Krzycki, J. A., Kolodziej, A. F., Alex, L., Orme-Johnson, W. H., & Walsh, C. T. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3031.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.
Schauer, N. L., & Ferry, J. G. (1982) *J. Bacteriol. 150*, 1.
Schauer, N. L., & Ferry, J. G. (1986) *J. Bacteriol. 165*, 405.
Schonheit, P., Moll, J., & Thauer, R. R. (1979) *Arch. Microbiol. 123*, 105.
Scott, R. A., Wallin, S. A., Czechowski, M., DerVartainian, D. V., LeGall, J., Peck, H. D., Jr., & Moura, I. (1984) *J. Am. Chem. Soc. 106*, 6864.
Shuber, A. P., Orr, E. C., Recny, M. A., Schendel, P. F., May, H. D., Schauer, N. L., & Ferry, J. G. (1986) *J. Biol. Chem. 261*, 12942.
Smith, G. E., & Summers, M. D. (1980) *Anal. Biochem. 109*, 123.
Southern, E. M. (1979) *J. Mol. Biol. 98*, 503.
Tabor, S., & Richardson, C. C. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1074.
Tan, S. L., Fox, J. A., Kojima, N., Walsh, C. T., & Orme-Johnson, W. H. (1984) *J. Am. Chem. Soc. 106*, 306.
Tanaka, M., Haniu, M., Matsueda, G., Yasunobu, K. T., Himes, R. H., Akagi, J. M., Barnes, E. M., & Devanathan, T. (1971) *J. Biol. Chem. 246*, 3953.
Tanaka, M., Haniu, M., Yasunobu, K. T., Himes, R. H., & Akagi, J. M. (1973) *J. Biol. Chem. 248*, 5215.
Voordouw, G., Menon, N. K., LeGall, J., Choi, E. S., Peck, H. D., Jr., & Przybyla, A. E. (1989) *J. Bacteriol. 171*, 2894.
Wackett, L. P., Hartweig, E. A., King, J. A., Orme-Johnson, W. H., & Walsh, C. T. (1987) *J. Bacteriol. 169*, 718.
Walsh, C. T. (1986) *Acc. Chem. Res. 19*, 216.
Weil, C. F., Cram, D. S., Sherf, B. A., & Reeve, J. N. (1988) *J. Bacteriol. 170*, 4718.
Weirenga, R. K., & Hol, W. G. J. (1983) *Nature 302*, 842.
Yanisch-Perron, C., Viera, J., & Messing, J. (1985) *Gene 33*, 103.

Spontaneous Incorporation of Bacteriorhodopsin into Large Preformed Vesicles†

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ABSTRACT: Bacteriorhodopsin, either as purple membrane sheets or as detergent-solublized protein, was found to incorporate spontaneously into both large unilamellar vesicles (LUVs) and sized multilamellar vesicles (MLVs) under either gel-phase or liquid-phase conditions. These results were obtained with LUVs of various lipid compositions, including dimyristoylphosphatidylcholine (DMPC), DMPC and cholesterol, dioleoylphosphatidylcholine (DOPC), and DOPC and cholesterol. The lipid to protein (L/P) ratio of all proteoliposomes arising from these preformed vesicles continually increased in the presence of protein-free vesicles. Under fluid-phase conditions, the mixing of LUVs of DMPC with proteoliposomes showed vesicle growth independent of lipid concentration, suggesting that growth was due to lipid transfer. However, under gel-phase conditions a more rapid, concentration-dependent increase in the L/P ratio of the proteoliposome was observed, suggesting that growth occurred by a mechanism other than lipid transfer from vesicles to proteoliposomes. The use of the proteoliposome as an artificial lipid–protein membrane model is discussed.

Integral membrane proteins spontaneously insert into preformed small unilamellar vesicles (SUVs)[1] to form a variety of protein–lipid bilayer vesicles, designated proteoliposomes (Scotto & Zakim, 1985, 1986, 1988; Scotto et al., 1987). Several different integral membrane proteins have been incorporated by this procedure: these include bacteriorhodopsin, as crystalline arrays or detergent-solubilized monomers, UDP-glucuronosyltransferase, cytochrome oxidase (Scotto & Zakim, 1985, 1988), and the thrombospondin receptor (Berk et al.,

† This work was supported by a grant from the National Institutes of Health (GM-36651). Portions of this work have been presented at the 32nd Annual Meeting of the Biophysical Society, Phoenix, AZ, Feb 1988.
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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; MLVs, multilamellar lipid vesicles; LUVs, large unilamellar vesicles; SUVs, small unilamellar lipid vesicles.

0006-2960/90/0429-7244$02.50/0

1989). This technique, therefore, is widely applicable to the reconstitution of membrane proteins. Neither soluble proteins nor peripheral membrane proteins (i.e., aldolase, hemoglobin, bovine serum albumin, and cytochrome *c*) interact with preformed vesicles, nor do they interfere with the insertion of integral membrane proteins under any of the conditions examined. The reconstituted membrane proteins are inserted in a nonrandom direction, and enzyme function is restored; in the case of bacteriorhodopsin, we were able to reconstitute a functional light-drive proton pump in a closed vesicle (Scotto & Zakim, 1985, 1986, 1988; Scotto et al., 1987).

The lipid to protein (L/P) ratio of proteoliposomes can be increased by allowing the nascent proteoliposomes to interact with protein-free vesicles. This occurs more rapidly under gel-phase conditions than under liquid-crystalline conditions. We have shown previously that the former proceeds predominantly by a vesicle to vesicle fusion process whereas the mechanism of the increase in lipid content of the proteoliposomes under fluid-phase conditions is unclear. Furthermore, additional integral membrane proteins can be inserted into the nascent proteoliposomes to form complex protein compositions within a given vesicle (Scotto et al., 1987).

Although purified integral membrane proteins spontaneously incorporate into small unilamellar vesicles (SUVs), little is known about the application of this process to large vesicles. In fact, our earlier observations suggested that the incorporation process was limited to SUVs, since large unilamellar vesicles (LUVs) prepared by the fusion of SUVs did not seem to incorporate integral membrane proteins (Scotto & Zakim, 1985). However, the formation of large vesicles by gel phase induced vesicle fusion is difficult to control and often leads to the formation of LUVs as well as unusual multilamellar vesicles (MLVs). Additionally, our recent work with fluid-phase SUVs has indicated that longer time periods were required in some instances to effect a spontaneous insertion of a membrane protein (Scotto & Zakim, 1985, 1988). Therefore, we have extended our examination to large, more planar membranes in order to determine whether spontaneous incorporation occurs in membranes that are more closely related to those found in cells and organelles, as has been previously suggested (Engelman 1982). Unlike SUVs prepared by sonication, which are metastable structures due to their severe surface curvature and thus prone to vesicle to vesicle fusion (Kantor & Prestegard, 1978; Wong et al., 1982), both sized LUVs and sized MLVs prepared by extrusion are more stable bilayer structures (Hope et al., 1985; Mayer et al., 1986).

The ability of bacteriorhodopsin, either as small crystalline arrays or as monomers produced by detergent solubilization, to spontaneously insert into the outher lamellar of a series of sized vesicles was examined, in both fluid-phase and gel-phase vesicles. The data presented in this report indicate that the process of spontaneous incorporation of bacteriorhodopsin is not limited to SUVs. The spontaneous incorporation of at least one integral membrane protein occurs independently of the curvature of the bilayer. We also discuss possible mechanisms involved in the increase in the lipid content of the proteoliposomes and the potential role of proteoliposomes as a lipid–protein membrane model.

Materials and Methods

Materials. DMPC and DOPC were purchased from Avanti, Birmingham, AL. All other chemicals used were the best available commercial grades.

Preparation of SUVs Containing DMPC or DOPC. Phospholipid (70 mg) in chloroform was added to a stainless steel tube and solvent removed under a stream of dry nitrogen. When vesicles containing cholesterol were prepared, cholesterol dissolved in chloroform (10 mg/mL) was added to a stainless steel tube and mixed with the phospholipid. Solvent was removed under a stream of dry nitrogen, leaving a film of cholesterol and DMPC on the vessel wall. The dried residue was further dried under vacuum for 1 h and then hydrated for 1 h in 50 mM Hepes, pH 7.5, 100 mM KCl, 0.1 mM EDTA, and 0.02 mM azide. The mixture was sonicated in a stainless steel tube suspended in a water bath at either 4 °C for DOPC or 37 °C for DMPC. Sonication was carried out for 30 min with a Heat Systems W225 sonicator, under a stream of dry argon. The standard tip was used at 40% of maximum power; the output was pulsed so that power was delivered for 60% of the total time of sonication. These precautions were taken to prevent degradation of the phospholipid during sonication (Hauser, 1971). Samples appeared clear at the end of sonication. Sonicated vesicles were centrifuged at 37 °C (4 °C for DOPC) for 30 min at 39 000 rpm in a Beckman 40Ti rotor to remove titanium particles and any multilamellar vesicles remaining at the end of the sonication.

Preparation of Sized LUVs and MLVs of DMPC or DOPC. To prepare various-size vesicles, MLVs (10 mg/mL) of the required lipid composition were dried from solvent as described above and were subsequently hydrated by 10 cycles of freezing and thawing. The MLVs were then extruded through defined-size polycarbonate membranes 10 times per each "sized" preparation in a LIPEX extruder [essentially as described by Hope et al. (1985) and Mayer et al. (1986)]. These "sized" LUVs and MLVs were maintained at 37 °C.

Characterization of Newly Formed Proteoliposomes. From previous work with SUVs it is known that the formation of proteoliposomes from preformed vesicles occurs in two steps. First, an integral membrane protein spontaneously incorporates into a preformed vesicle, and second, there is a time-related increase in the lipid content of the proteoliposome from interactions with the remaining protein-free vesicles. During the second step there is a "growth" of the proteoliposome by the addition of lipid by either lipid transfer or membrane fusion; in SUVs, the actual mechanism depends upon the phase of the participating bilayers. Due to the rate of membrane fusion between SUVs and proteoliposomes these steps cannot always be completely separated and are best measured as the L/P ratio of the complex following equilibrium density centrifugation, during which the lipid–protein vesicles are separated from protein-free vesicles and any unincorporated protein. In order to avoid the difficulties of distinguishing between the spontaneous insertion of bacteriorhodopsin and a detergent-catalyzed insertion of the protein, the majority of these experiments were performed with small crystalline arrays of bacteriorhodopsin. The purple membrane isolated according to Oesterhelt and Stoeckenius (1974) was sonicated at 4 °C to reduce the size of the crystalline arrays of bacteriorhodopsin so that they do not sediment under the usual *g* force necessary to isolate purple membrane. This decrease in size of the purple membrane does not change the phytolipid content of these aggregates, and thus, a residual 7–11 molecules of lipid remain per monomer of bacteriorhodopsin. Therefore, a phosphorus content of a putative reconstitution of greater than 11 mol/mol is necessary to indicate the formation of a lipid–protein vesicle (Scotto & Zakim, 1985, 1988). These smaller arrays of bacteriorhodopsin result in a tight banding of the nascent proteoliposomes, i.e., purple bands of proteoliposomes 12 mm in width or less in many cases. In fact, the band width is closely related to the secondary process, growth of the proteoliposome. Initially, the nascent proteoliposomes form a very

tight band reflecting a narrow L/P ratio; as the lipid content increases, this band widens, and a broader L/P ratio is contained within the band. Representative experiments are presented in the tables. All experiments were either duplicated or triplicated to confirm the incorporations presented and the rates of increase of lipid content of the nascent proteoliposomes.

Density Gradient Centrifugation. Lipid vesicles, lipid–protein complexes (proteoliposomes), and proteins were separated from each other by centrifugation on glycerol gradients containing 10 mM Hepes, pH 7.5, and 100 mM KCl (Scotto & Zakim, 1988). The compositions of the gradients are given in the text and legends to the figure and tables. The gradients contained a 0.5-mL "cushion" of fluoroinert FC-40. Equilibrium conditions were achieved unless noted. Gradients were either side punctured to remove the visible band or fractionated from the top with an ISCO Model 185 density gradient fractionator.

Purification of Bacteriorhodopsin. Halobacterium halobium strain E1 was a generous gift of Drs. W. Stoeckenius and R. Bogomolni. The bacteria were grown and the membranes purified according to Oesterhelt and Stoeckenius (1974). The purple membrane was separated from the red membrane by density gradient centrifugation on sucrose. The ratio of OD_{260}/OD_{560} was between 2.0 and 2.1 for all preparations of bacteriorhodopsin. Bacteriorhodopsin monomers were prepared by solubilizing the purple membrane with octyl glucoside in 50 mM Mops, pH 6.9, at a detergent to protein ratio of 10, while shaking for 24 h in the dark (Dencher & Heyn, 1980). After this treatment, all the bacteriorhodopsin remained in the supernatant when centrifuged for 45 min at 200000*g*.

Analytical Methods. Proteins were measured by coprecipitation with S-RNA (Polacheck & Cabib, 1981) followed by protein determination with bicinchoninic acid (Smith et al., 1985). Phospholipids were measured by a modification (Scotto & Zakim, 1985) of the malachite green method of Chalvardjian and Rudniski (1970). Analytical assays were performed in duplicate or triplicate on each sample.

Results

Incorporation of Bacteriorhodopsin into Large Preformed Vesicles. To determine the effect of the surface curvature on the spontaneous incorporation of bacteriorhodopsin into preformed vesicles, a graduated series of "sized" liposomes was prepared by the extrusion technique (Hope et al., 1985; Mayer et al., 1986). This procedure can be used to produce a incremental series of large unilamellar vesicles (LUVs) ranging from 0.05 to 0.1 μm. Vesicles of larger sizes (up to 10 μm) prepared by this technique are sized multilamellar vesicles (MLVs). These vesicles were mixed with purified bacteriorhodopsin. The data in Table I, describing L/P ratios of the isolated proteoliposomes, show that within 2 h there was a spontaneous incorporation of bacteriorhodopsin into large fluid-phase vesicles ranging in size from 0.1 to 1.0 μm. Increasing the time of incubation of the mixture of bacteriorhodopsin and vesicles resulted in a small increase in the lipid to protein ratio of the nascent proteoliposomes as well as the insertion of bacteriorhodopsin into slightly larger vesicles of up to 3 μm in size. Bacteriorhodopsin also spontaneously inserts into gel-phase LUVs and into MLVs of 0.1–10 μm within 30 min (data not shown). However, no rapid increase in the L/P ratio of the proteoliposomes was found in this experiment, as had been observed when the protein-free vesicles in the mixture were gel-phase SUVs.

Effect of Vesicle Concentration on the Growth of Proteoliposomes from LUVs. The rapid increase in the lipid content of proteoliposomes from gel-phase protein-free SUVs is due to vesicle fusion (Scotto & Zakim 1985, 1986). However, the slower increase in the lipid content of the proteoliposome when the protein vesicles are LUVs indicates that vesicle fusion may not be the major pathway of growth during this second step in the formation of proteoliposomes. There are two significant differences between SUVs and large lipid vesicles which may affect their interaction with proteoliposomes: the multilamellar nature of most of the sized vesicles and the reduction in the concentration of vesicles at similar phospholipid concentrations. We have therefore further examined the effect of the phase of the protein-free vesicles on the rate of increase in lipid content of proteoliposomes with respect to these two variables. Both 0.1-μm LUVs and 1.0-μm MLVs incorporated bacteriorhodopsin spontaneously, thereby allowing for a comparison of the effect of unilamellar vesicles versus the MLV on the rate of increase in the lipid content of the proteoliposome. The effect of varying the phospholipid concentration for both 0.1-μm LUVs and 1.0-μm MLVs is shown in Table II. Under fluid-phase conditions proteoliposomes formed initially from 0.1-μm LUVs do not increase in lipid content in response to as much as a 10-fold increase in vesicle concentration. However, under gel-phase conditions increasing the vesicle concentration does affect the growth of the proteoliposome. As shown in Table II, increasing the vesicle concentration of LUVs causes a direct increase in the lipid content of the proteoliposome, resulting in a vesicle with a 4–5-fold higher lipid content than that obtained at the lowest phospholipid concentration.

The effect of the increased size and the multilamellar nature of larger phospholipid vesicles on the interaction between proteoliposomes and protein-free vesicles was examined with 1.0-μm vesicles of DMPC as was previously done with the 0.1-μm LUVs. As shown in Table II, varying the concentration of the 1.0-μm fluid-phase vesicles of DMPC did not affect the rate of increase of the lipid content of the proteoliposomes. In fact, the lipid to protein ratios were similar to that obtained with the 0.1-μm LUVs. This result could be viewed as unusual because of the difference in size of the

Table I: Spontaneous Incorporation of Bacteriorhodopsin into Large DMPC Vesicles[a]

vesicle size (μm)	L/P of isolated bacteriorhodopsin complexes 2 h	24 h	96 h
0.1	28	103	105
0.2		89	78
0.4	18	53	48
0.6	21	62	37
0.8	20	38	41
1.0	20	31	40
2.0	11*	18	24
3.0	10*	18	21
5.0	*	11*	11*
8.0	*	*	*
10.0	*	*	*

[a] Bacteriorhodopsin (1 mg) was mixed with large liquid-phase preformed vesicles comprised of DMPC (5 mg); mixtures of these two components were incubated at 37 °C for 2–96 h. The mixtures were separated by equilibrium density gradient centrifugation on 10–85% glycerol density gradients as described under Materials and Methods. The bacteriorhodopsin–lipid complexes were removed by side puncture of the tube and L/P ratios of the samples were determined as described under Materials and Methods. (*) Asterisk represents unincorporated bacteriorhodopsin which sedimented to an identical point as bacteriorhodopsin which was not mixed with lipid vesicles. L/P ratios of greater than 10–11* represented an increase in the content of lipid in the purified bacteriorhodopsin and are indicative of a stable lipid–protein complex (for details, see Materials and Methods).

Table II: Effect of Lipid Concentration on Bacteriorhodopsin Incorporation and Vesicle Growth[a]

	L/P of isolated bacteriorhodopsin complexes					
	0.1-μm LUVs			1.0-μm MLVs		
lipid (mg/mL)	37 °C 1 h	20 °C 0.5 h	5 °C 0.5 h	37 °C 1 h	20 °C 0.5 h	5 °C 0.5 h
5	24	19	30	22	21	48
10	24	23	40	19	130	37
20	24		60	16	108	79
30	29	65	82	18	231	108
40	30	91	102	15	240	375
50	30	105	136	15	501	981

[a] Bacteriorhodopsin (1 mg) was mixed with increasing amounts of DMPC vesicles as either 0.1-μm LUVs or 1.0-μm MLVs, from 5 to 50 mg of phospholipid/mL, and the mixtures were incubated above or below the phase transition for the periods indicated. The mixtures were separated by equilibrium density gradient centrifugation on linear glycerol density gradients as described under Materials and Methods. The bacteriorhodopsin–lipid complexes (proteoliposomes) were removed by side puncture of the tube, and L/P ratios of the samples were determined as described under Materials and Methods.

Table III: Spontaneous Incorporation of Bacteriorhodopsin into Large Unilamellar DMPC Vesicles[a]

	L/P of isolated bacteriorhodopsin complexes			
	fluid phase		gel phase	
vesicle size (μm)	1 h	3 h	10 min	30 min
sonicated ~0.02	32	54	156	258
extruded 0.05	26	34	34	41
0.08	25	36	39	46
0.1	26	33	33	49

[a] Bacteriorhodopsin (1 mg) was mixed with SUVs or LUVs comprised of DMPC (5 mg) at the liquid phase. The mixtures were incubated above or below the phase transition for the periods indicated. The mixtures were then separated by equilibrium density gradient centrifugation on 10–85% glycerol density gradients as described under Materials and Methods. Bacteriorhodopsin–lipid complexes, proteoliposomes, were removed by side puncture of the tube, and L/P ratios of the samples were determined as described under Materials and Methods.

vesicles. Since the MLV has a greater lipid content of the outer bilayer into which the bacteriorhodopsin inserts, the L/P ratio of the nascent proteoliposome should be higher than that of an LUV which incorporated bacteriorhodopsin. However, the L/P ratio of the isolated proteoliposomes is not due only to the initial protein insertion event but also to the spontaneous and rapid insertion of additional protein into the nascent proteoliposome. The insertion of an integral membrane protein into a protein-free bilayer creates a vesicle that is more likely to incorporate the additional protein than the remaining protein-free vesicles (Scotto & Zakim, 1985, 1988). This is evident when the equilibrium density gradients were observed; the resolution of proteoliposomes isolated immediately after formation consistently demonstrated a tight banding; similar results have been previously obtained with SUVs (Scotto & Zakim, 1985, 1988). After extended growth, the banding of the proteoliposomes isolated on the gradients broadened, and thus, the L/P ratio averages a distribution of proteoliposomes. Thus, the propensity of bacteriorhodopsin, like other integral membrane proteins, to preferentially insert into a lipid–protein bilayer in favor of a protein-free lipid bilayer causes the nascent protein-containing membrane to be packed with the maximal amount of bacteriorhodopsin possible (in the case of bacteriorhodopsin this is a 11–15 L/P ratio) before other protein-free bilayers are utilized and before measurable growth in the lipid content occurs. This interpretation of the insertion process can be explained by the results of the addition of unincorporated bacteriorhodopsin to a mixture of proteoliposomes with a high L/P ratio and protein-free vesicles, either SUVs (Scotto et al., 1987) or LUVs. The high L/P ratio of the original proteoliposomes decreased as the new bacteriorhodopsin was inserted, indicating that the newly added bacteriorhodopsin incorporated preferentially into the proteoliposomes and not the protein-free vesicles. No newly formed proteoliposomes were detected in either experiment [Scotto et al. (1987) and data not shown].

In addition to the difference in size of the two populations of vesicles, the presence of the multiple bilayers of the fluid-phase protein-free MLVs does not cause an apparent difference in the growth of the proteoliposome. However, in contrast to these results obtained with either fluid-phase LUVs or fluid-phase MLVs, the data obtained with gel-phase MLVs, at either 20 or 5 °C, revealed a different pattern. The rapid augmentation in lipid content of the gel-phase proteoliposomes in the presence of high concentrations of 1-μm vesicles increased with higher vesicle concentrations, and the increase was 5–8-fold greater than that observed with the 0.1-μm LUV preparations (Table II). This more rapid increase in L/P ratio in the presence of 1.0-μm MLVs must be a consequence of either the multilamellar nature of the proteoliposomes prior to separation or the presence of protein-free MLVs.

Effect of Size of Unilamellar Vesicles on the Incorporation of Bacteriorhodopsin and Proteoliposome Growth. Although both SUVs prepared by sonication (~0.02 μm) and 0.1-μm LUVs prepared by extrusion incorporate bacteriorhodopsin spontaneously, these vesicles behave differently with respect to the growth or increase in lipid content of the proteoliposome. The rapid increase of lipid content of proteoliposomes mixed with gel-phase SUVs due to the fusion of SUVs with the proteoliposomes (Scotto & Zakim, 1985, 1988) has not been observed with sized vesicles of 0.1-μm and larger. In order to further evaluate this difference, LUVs smaller than 0.1 μm were prepared by extrusion. These vesicles were either 0.05 or 0.08 μm in size, with a higher degree of bilayer curvature than the 0.1-μm LUVs. SUVs formed by sonication are the smallest vesicles available and thus have the greatest degree of curvature that can be obtained in a lipid vesicle, and were used for comparison. The interactions of proteoliposomes formed from each size vesicle with the similar-size protein-free vesicles were examined under both fluid- and gel-phase conditions to determine if a smaller size unilamellar vesicle, with a curvature intermediate between those of SUVs and 0.1-μm LUVs, would allow for a rapid growth of the proteoliposomes by vesicle fusion.

As shown in Table III, bacteriorhodopsin incorporated into all size vesicles tested. The differences in the extent of increase in the lipid content of the proteoliposomes with time indicate, however, that there are differences in the way these proteoliposomes interact with the remaining protein-free vesicles. Proteoliposomes in the presence of fluid-phase SUVs increased in lipid content more rapidly than with any of the sized extruded vesicles. However, under gel-phase conditions a large difference was observed between the sonicated and extruded vesicle preparations in both the extent of lipid content and the rate of increase with time. SUVs caused a rapid increase in the L/P ratio of the proteoliposomes. Moreover, no significant difference was observed between the three sizes of LUVs prepared by extrusion regardless of the increased curvature. Although the extent of curvature of DMPC bilayers did not restrict the spontaneous insertion of bacteriorhodopsin, the second step of proteoliposome formation appeared to be sig-

nificantly affected by this physical property. The smallest vesicle SUVs prepared by sonication (~0.02 μm) are the least stable form of unilamellar vesicle and have been shown to be capable of fusion with other vesicles regardless of their curvature (Scotto & Zakim, 1988). Larger vesicles, such as the LUVs prepared by extrusion (≥0.05 μm) did not demonstrate this instability when interacting with other preformed bilayer or lipid–protein vesicles but appeared to be more stable and interact with other membranes more slowly. In fact, the growth of the proteoliposomes observed with protein-free LUVs is consistent with the rates of phospholipid transfer (Roseman & Thompson, 1980; Jones & Thompson, 1989).

Incorporation of Noncrystalline Arrays of Bacteriorhodopsin into LUVs and *MLVs of DMPC.* A variety of integral membrane proteins have been spontaneously inserted into preformed SUVs both in the absence of any detergent and with solubilized membrane proteins with only residual or low levels of detergent (Scotto & Zakim, 1985, 1988; Scotto et al., 1987; Berk et al., 1989). The data from these prior studies established that the spontaneous insertion of an integral membrane protein is not related to either a particular protein, the presence of the detergent, or the type of aggregation of the protein but rather is a manifestation of the inherent ability of bilayers to interact preferentially and in a nonrandom fashion with the hydrophobic moieties of integral membrane proteins. Although the hydrophobic regions of the isolated membrane proteins are undoubtly shielded from the aqueous solvent, this shielding is either not complete or in a dynamic flux which exposes some portion of this hydrophobic property, thereby allowing for the insertion event. The present study has taken advantage of these finding to use the small crystalline arrays of bacteriorhodopsin as a model to study protein insertion. However, an alternative interpretation of these events is that the bacteriorhodopsin does not insert but fuses as a planar membrane with the bilayer and thus does not reflect any true interaction between the membrane protein and the bilayer. To clarify whether the insertion of bacteriorhodopsin as detergent-free crystalline arrays is similar to that of soluble membrane protein, bacteriorhodopsin was solubilized in octyl glucoside to produce monomers that were isolated from excess detergent on sucrose density gradients containing sufficient detergent to keep the protein soluble; some bacteriorhodopsin was also isolated on density gradients containing no added detergent to isolate small aggregates of this protein. When mixed with preformed 0.1-μm LUVs of DMPC, bacteriorhodopsin in both forms incorporated in a manner similar to that of the detergent-free crystalline arrays of purple membrane (data not shown). Bacteriorhodopsin prepared in this manner also rapidly inserted into large MLVs, unlike the small crystalline arrays of bacteriorhodopsin used in the experiments presented in Table I (data not shown). This latter result is consistent with our earlier findings that the larger aggregates of bacteriorhodopsin and detergent-depleted UDP-glucuronosyltransferase incorporated more slowly than smaller aggregates or detergent-"soluble" protein (Scotto & Zakim, 1988). Bacteriorhodopsin incorporated both in the absence and in the presence of detergent, indicating that the detergent does not facilitate the insertion of the bacteriorhodopsin. Since the presence of detergent in the proteoliposome can affect the rate of phospholipid transfer, no quantitation of the changes in the L/P ratio of the vesicles were performed.

Effect of Cholesterol on Incorporation and Proteoliposome Growth. Cholesterol is known to perturb the packing of the gel phase of DMPC and to cause fusion of SUVs with each other as well as with proteoliposomes (Scotto & Zakim, 1986).

Table IV: Effect of Lipid Concentration on Bacteriorhodopsin Incorporation and Vesicle Growth—0.1-μm Vesicles of DMPC and Cholesterol[a]

	L/P of isolated bacteriorhodopsin complexes		
lipid (mg/mL)	37 °C 2 h	21 °C 0.5 h	5 °C 0.5 h
5	28	19	14
10	27	27	33
20	30	22	50
30	31	15	77
40	34	17	125
50	32	19	181

[a] Bacteriorhodopsin (1 mg) was mixed with increasing amounts of 0.1-μm LUVs of DMPC and 12 mol % cholesterol, from 5 to 50 mg, and the mixtures were incubated both above and below the phase transition for the periods indicated. The mixtures were separated by equilibrium density gradient centrifugation on linear glycerol density gradients as described under Materials and Methods. The bacteriorhodopsin–lipid complexes were removed by side puncture of the tube, and L/P ratios of the samples were determined as described under Materials and Methods.

We have, therefore, examined the effect of cholesterol on the incorporation of bacteriorhodopsin and the growth of the proteoliposome in large vesicles of DMPC. Fluid-phase vesicles of DMPC ranging in size from 0.1 to 10 μm and containing 12 mol% cholesterol spontaneously incorporated bacteriorhodopsin similarly to vesicles of pure DMPC (data not shown). The effect of cholesterol on 0.1-μm LUVs was examined as a function of both lipid phase and vesicle concentration. As shown in Table IV, the lipid content of the proteoliposome was not affected by the concentration of fluid-phase LUVs. Additionally, there was no effect on the interaction of proteoliposomes and LUVs containing cholesterol at 21 °C. This is somewhat surprising since 21 °C is the temperature at which a maximal rate of vesicle fusion with SUVs was found (Scotto & Zakim, 1986). However, when the vesicles were well within the gel phase at 5 °C, a direct correlation was observed between increased L/P ratios of the isolated proteoliposomes and increased vesicle concentration. These rates of increase were similar to those obtained with LUVs of pure DMPC (Table II). Thus, unlike SUVs, LUVs of DMPC containing lipid "impurities" like cholesterol do not allow for the rapid fusion of the lipid vesicles with proteoliposomes.

Gel-phase SUVs have been previously shown to incorporate integral membrane proteins more rapidly than fluid-phase vesicles (Scotto & Zakim, 1986, 1988). The incorporation of bacteriorhodopsin into 0.1-μm gel-phase LUVs of DMPC containing 12 mol % cholesterol at 21 °C was complete within 1 min; the isolated proteoliposome had a lipid to protein ratio of 30. However, no significant increase in the L/P ratio of the proteoliposomes was observed during 1 h of incubation at 21 °C.

Effect of Phospholipid Unsaturation on the Incorporation of Bacteriorhodopsin into LUVs. The spontaneous incorporation of integral membrane proteins into preformed SUVs is not limited to disaturated phosphatidylcholines but also occurs in fluid-phase bilayers of DOPC (Scotto & Zakim, 1988). Therefore, we examined the insertion of bacteriorhodopsin into large vesicles of DOPC of various sizes. Shown in Table V are the L/P ratios of proteoliposomes obtained by mixing bacteriorhodopsin with preformed large lipid vesicles of DOPC for 24 h. In other experiments we confirmed that the actual insertion into preformed vesicles was effected in a period similar to that observed with DMPC (data not shown). These results are similar to those obtained for the large vesicles

Table V: Spontaneous Incorporation of Bacteriorhodopsin into Large DOPC Vesicles[a]

vesicle size (μm)	L/P of isolated bacteriorhodopsin complexes at 24 h	vesicle size (μm)	L/P of isolated bacteriorhodopsin complexes at 24 h
0.1	30	1.0	51
0.2	23	2.0	41
0.4	34	3.0	39
0.6	38	5.0	44
0.8	70	8.0	54

[a] In order to examine the interaction of bacteriorhodopsin (1 mg) with large liquid-phase preformed vesicles comprised of DOPC (5 mg), mixtures of these two components were incubated at 37 °C for 24 h. The mixtures were separated by equilibrium density gradient centrifugation on 10–85% glycerol density gradients as described in Table I. The isolated bacteriorhodopsin complexes were removed and analyzed as described in Table I.

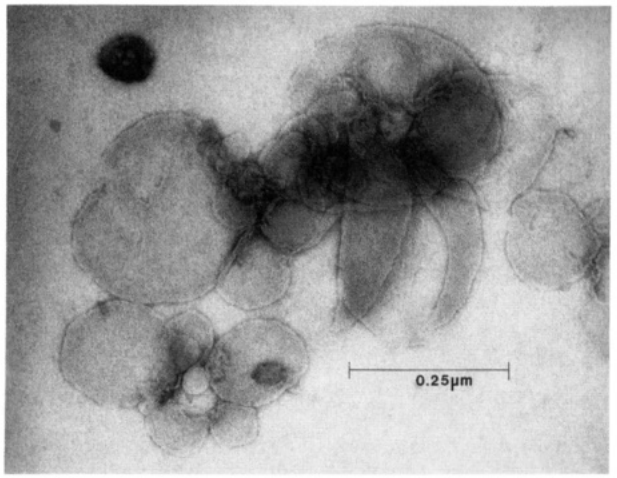

FIGURE 1: Electron microscopy of bacteriorhodopsin after incorporation into LUVs of DOPC. Bacteriorhodopsin was mixed with LUVs of DOPC at 37 °C for 1 h. The mixture was separated by 10–75% glycerol density centrifugation for 36 h at 25 000 rpm in an SW-41 rotor. The lipid–protein vesicles were removed by side puncture and dialyzed to remove the glycerol. The L/P ratio of the isolated lipid–protein vesicles was 25. Samples were prepared for electron microscopy by negative staining, with uranyl acetate of samples adhered to glow-discharged carbon film grids.

of DMPC shown in Table I and for those comprised of DMPC and cholesterol (data not shown). The only apparent difference is that all sizes of the large vesicles of DOPC readily incorporated bacteriorhodopsin, whereas only vesicles of DMPC of 3 μm or less were capable of incorporating this membrane protein. This result does not reflect a difference in the behavior of the vesicles composed of these two lipids but rather is a consequence of a smaller particle size of the purified bacteriorhodopsin used for these studies with DOPC. In a separate experiment 10-μm MLVs of DMPC were found to easily incorporate this preparation of bacteriorhodopsin.

Electron Microscopy of Bacteriorhodopsin–DOPC Proteoliposomes. The proteoliposomes formed from mixing bacteriorhodopsin with 0.1-μm LUVs for 1 h were isolated from the remaining LUVs by equilibrium density centrifugation on a 10–75% glycerol gradient, the glycerol was removed by dialysis, and the proteoliposomes were examined by electron microscopy. The electron micrograph in Figure 1 demonstrates the vesicular nature of these lipid–protein complexes. Additionally, these proteoliposomes exhibit a heterogeneity of size, ranging from 0.07 to 0.3 μm. This heterogeneity is due to the insertion of the crystalline arrays of bacteriorhodopsin; the size of these arrays is variable. The smaller size of some of the vesicles may be an artifact occurring

Table VI: Spontaneous Incorporation of Bacteriorhodopsin into Unilamellar DOPC–Cholesterol Vesicles[a]

	L/P of isolated bacteriorhodopsin complexes			
vesicle size (μm)	1 min	10 min	1 h	3 h
sonicated ~0.02	30	41	53	62
extruded 0.05	14	20	24	23
0.08		16	23	22
0.1	11*	18	18	19

[a] In order to examine the interaction of bacteriorhodopsin (1 mg) with large unilamellar preformed vesicles comprised of DOPC containing 12 mol % cholesterol (5 mg), mixtures of these two components were incubated at the temperatures and times indicated. The mixtures were separated by equilibrium density gradient centrifugation on 10–85% glycerol density gradients and analyzed as described in Table I.

during isolation of the proteoliposomes. For example, some of the larger proteoliposomes may be reduced in size by the shear forces exerted on the vesicles when they were collected by side puncture of the gradient with a hypodermic needle. This would not be unlike the extrusion process used for the formation of the LUVs.

Effect of the Addition of Cholesterol to Bilayers of DOPC on the Formation of Proteoliposomes. Cholesterol, which affects the interaction of proteoliposomes and SUVs, does not affect the gel-phase interaction of LUVs of DMPC and proteoliposomes (Table VI). To determine whether the inclusion of cholesterol, which changes the packing of phospholipid bilayers (Lippert & Peticolas, 1971; Kutchari et al., 1983), affects either the insertion of bacteriorhodopsin into vesicles of reduced curvature or the growth of the proteoliposome, unilamellar vesicles of DOPC containing 12 mol % cholesterol were prepared. The data shown in Table VI demonstrate that LUVs prepared by extrusion incorporated bacteriorhodopsin more slowly than SUVs (i.e., 0.1-μm LUVs did not incorporate bacteriorhodopsin within the first minute). Futhermore, examination of results from the first two time periods showed that smaller LUVs more rapidly inserted bacteriorhodopsin. The differences between the proteoliposomes formed from the different-size LUVs were rapidly masked, however, by the increase in lipid content, such that this difference was no longer apparent by 10 min. In the case of SUVs, it appears from the extent of growth of the proteoliposomes formed during the initial minute that insertion was more rapid than that observed with even the smallest LUV. Furthermore, the rate of lipid growth of the proteoliposomes mixed with SUVs exceeds that measured for any of the LUVs.

Discussion

The purpose of this and prior work from this laboratory has been 2-fold. First, we have examined the spontaneous incorporation of integral membrane proteins into the matrix of lipid bilayers and the bilayer conditions which either overcome or maintain the barrier (kinetic, hydrodynamic, and/or thermodynamic) for this process. Second, we have used these conditions to construct a proteoliposome to serve as an artificial lipid–protein membrane model for the study of membrane events. In order to further evaluate the characteristics of the barrier to the facile insertion of integral membrane proteins into the bilayer, we have examined the effect of the radii of curvature of the lipid vesicle on protein incorporation. It has been shown that the highly curved bilayer of SUVs is prone to both stress and packing irregularities (Lawaczeck et al., 1975, 1976; Blaurock & Gamble, 1979). We have previously proposed that the thermodynamic barrier for the insertion of

a membrane protein into SUVs is overcome by the "fusion" of the hydrophobic moiety of an integral membrane protein with the bilayer at a region of reduced hydration presumably caused by a transient packing defect in the bilayer (Scotto & Zakim, 1988). Results in the present work indicate that these transient defects or packing irregularities which occur in all lipid bilayers, but are less pronounced in large diameter vesicles (Lee, 1975, 1977), may be the underlying mechanism for the spontaneous insertion of integral membrane proteins into bilayers of vesicles which have curvatures that are more like those of natural membranes.

The data in the present paper show that the spontaneous reconstitution of bacteriorhodopsin is not limited to SUVs and that both LUVs and MLVs, given time, will spontaneously reconstitute this membrane protein. In fact, the relaxation of the curvature of the bilayer does not render the bilayer of preformed vesicles incapable of spontaneously incorporating bacteriorhodopsin, either as small crystalline arrays or as detergent-monomerized protein. The insertion of bacteriorhodopsin into these various-sized MLVs as well as the 0.1-μm LUV represents the first extension of the spontaneous insertion of bacteriorhodopsin into preformed vesicles other than SUVs. The insertion of the protein into the LUVs is similar to that of SUVs since only a single bilayer is present in the vesicle. However, the size MLVs present a potentially different situation since several bilayers are present in each vesicle. Nevertheless, since the initial interaction between the membrane protein and the MLV is restricted to the outermost lamella of the vesicle, it is appropriate for the present studies to disregard the effect of the inner lamella. Thus, the inner bilayers may be a contributing factor to the increase in lipid content of the vesicle during the growth of the proteoliposome but are not important for the study of the effect of surface curvature on the interaction of bacteriorhodopsin and the outer bilayer. This conclusion is further substantiated by the low initial L/P ratios of the isolated proteoliposomes formed from sized MLVs, which indicate that the inner bilayers are not present after isolation. This suggests that inner bilayers are either involved in the initial formation of the proteoliposome or separated from the "outer" lipid–protein membrane during equilibrium density centrifugation. The latter explanation is consistent with both our current understanding of the process of spontaneous formation of the proteoliposome and the effect of centrifugation of proteoliposomes containing encapsulated materials on glycerol gradients (Scotto, unpublished data).

The facile insertion of bacteriorhodopsin, either as the crystalline array or as detergent-solubilized protein, into performed LUVs suggests that the insertion into preformed bilayers of DMPC and DOPC is an inherent property of these phospholipid membranes. The insertion of integral membrane proteins into vesicles of other lipid compositions is currently under investigation. Although the insertion of integral membrane proteins into SUVs is not dependent upon the phospholipid composition of the bilayer, we have found that spontaneous insertion of bacteriorhodopsin into LUVs depends upon the phospholipid composition of the membrane (Scotto, unpublished data).

The mechanism by which the lipid content of the proteoliposomes increases after their initial formation is not yet clear. The rates of increase in the L/P ratio are consistent with lipid transfer. It is interesting that the direction of the net transfer favors the proteoliposomes. In fact, phospholipid transfer is persumed to be the predominant mechanism of growth under fluid-phase conditions. We are presently determining whether the increase in lipid content of the proteoliposome is solely due to phospholipid transfer, whether by solvation or by vesicle collision, or a combination of phospholipid transfer and vesicle to vesicle fusion. Collision has been recently implicated in the transfer of phosphatidylcholine between liposomes at high lipid concentrations (Jones & Thompson, 1989). It should be noted that the direction of net transfer of lipid, regardless of the exact nature of the mechanism, mimics the usual behavior of liposomes with cellular membranes, suggesting that the introduction of an integral membrane protein into the phospholipid bilayer changes the stability of the lipid component of this artificial membrane to a closer approximation of that of a biological membrane. Under gel-phase conditions this process may be more complex. Therefore, some other mechanism, such as vesicle fusion or even an incomplete fusion event, may not only contribute but could play a major role in the growth of proteoliposomes in the presence of protein-free vesicles. Gel-phase vesicles of neutral phospholipids aggregate, and SUVs, even in the absence of impurities, fuse slowly to form 0.1-μm vesicles; it is believed that larger vesicles formed by gel-phase fusion of SUVs are unstable and collapse to re-form 0.1-μm vesicles (Wong & Thompson, 1982; Wong et al., 1982). Thus, it seems unlikely that aggregation or collisions between gel-phase LUVs will readily result in a productive fusion. However, proteoliposomes formed from SUVs have been shown to be capable of forming stable structures of more than a micron (Scotto & Zakim, 1985), and these lipid–protein vesicles may be able to fuse productively with LUVs.

The application of this work to the reconstitution of lipid–protein membranes has several advantages over those of the earlier studies with SUVs. Although it is likely that the same mechanism is responsible for the initial interaction of bacteriorhodopsin with preformed bilayers of both small and large vesicles, the subsequent interaction of the nascent proteoliposome with protein-free vesicles differs between SUVs and LUVs with respect to both the rates of change of lipid–protein ratios and the size of protoliposomes observed by electron microscopy. Micrographs of proteoliposomes formed from SUVs reveal vesicles of several microns in size (Scotto & Zakim, 1985, 1986), while the formation of proteoliposomes from LUVs resulted in vesicles more closely related to the initial diameter of the lipid vesicles (Figure 1). In fact, it is the ability to spontaneously insert bacteriorhodopsin and presumably other integral membrane proteins into LUVs to form proteoliposomes with controlled lipid to protein ratios that described the simple formation of a complex artificial membrane as a alternative model to the liposome for membrane studies.

Registry No. DMPC, 18194-24-6; DOPC, 4235-95-4; cholesterol, 57-88-5.

References

Berk, G. I., Asch, A. S., Scotto, A. W., Silverstein, R. L., & Nachman, R. L. (1989) *Clin. Res. 37*, 377a.

Blaurock, A. E., & Gamble, R. C. (1979) *J. Membr. Biol. 50*, 187–204.

Chalvardjian, A., & Rudniski, E. (1970) *Anal. Biochem. 36*, 225–226.

Engelman, D. M., & Steitz, T. A. (1981) *Cell 23*, 411–422.

Hauser, H. (1971) *Biochem. Biophys. Res. Commun. 45*, 1049–1055.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55–65.

Jones, J. D., & Thompson, T. E. (1989) *Biochemistry 28*, 129–134.

Kantor, H. L., & Prestegard, J. (1978) *Biochemistry 17*, 1790–1795.

Kutchari, H., Chandler, L. H., & Zavoico, G. B. (1983) *Biochim. Biophys. Acta 736*, 137-149.
Lawaczeck, R., Kainosho, M., Girardet, J.-L., & Chan, S. I. (1975) *Nature 256*, 584-586.
Lawaczeck, R., Kainosho, M., & Chan, S. I. (1976) *Biochim. Biophys. Acta 443*, 313-330.
Lee, A. G. (1975) *Prog. Biophys. Mol. Biol. 29*, 3-56.
Lee, A. G. (1977) *Biochemistry 16*, 835-841.
Lippert, J. L., & Peticolas, W. I. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1572-1576.
Mayer, L. D., Hope, M. J., & Cullis, P. R. (1986) *Biochim. Biophys. Acta 858*, 161-168.
Nayar, R., Hope, M., & Cullis, P. R. (1989) *Biochim. Biophys. Acta 986*, 200-206.
Oesterhelt, D., & Stoeckenius, W. (1974) *Methods Enzymol. 31*, 667-678.
Olson, F., Hunt, C. A., Szoka, F. C., Vail, W. J., & Papahadjopoulos, D. (1979) *Biochim. Biophys. Acta 557*, 9-23.
Polacheck, I., & Cabib, E. (1981) *Anal. Biochem. 117*, 311-314.
Roseman, M. A., & Thompson, T. E. (1980) *Biochemistry 19*, 439-443.
Scotto, A. W., & Zakim, D. (1985) *Biochemistry 24*, 4066-4075.
Scotto, A. W., & Zakim, D. (1986) *Biochemistry 25*, 1555-1561.
Scotto, A. W., & Zakim, D. (1988) *J. Biol. Chem. 263*, 18500-18506.
Scotto, A. W., Goodwin, D., & Zakim, D. (1987) *Biochemistry 26*, 833-839.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Malia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76-85.
Suurkuusk, J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., & Thompson, T. E. (1976) *Biochemistry 15*, 1393-1401.
Wong, M., & Thompson, T. E. (1982) *Biochemistry 21*, 4133-4139.
Wong, M., Anthony, F. H., Tillack, T. W., & Thompson, T. E. (1982) *Biochemistry 21*, 4126-4132.

Damage Repertoire of the *Escherichia coli* UvrABC Nuclease Complex Includes Abasic Sites, Base-Damage Analogues, and Lesions Containing Adjacent 5′ or 3′ Nicks†

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Received March 8, 1990; Revised Manuscript Received April 30, 1990

ABSTRACT: Using oligonucleotide synthesis, we demonstrate a rapid and efficient method for the construction of DNA duplexes containing defined DNA lesions at specific positions. These DNA lesions include apyrimidinic sites, reduced apyrimidinic sites, and base-damage analogues consisting of *O*-methyl- or *O*-benzylhydroxylamine-modified apyrimidinic sites. A 49 base pair DNA duplex containing these lesions was specifically incised by the UvrABC nuclease complex. The incision sites occurred predominantly at the eighth phosphodiester bond 5′ and the fifth phosphodiester bond 3′ to the lesion. Multiple incisions were observed 3′ to the lesion. The extent of DNA incisions was base-damage analogues > reduced apyrimidinic sites > apyrimidinic sites. Introduction of 3′ or 5′ nicks at the site of a base-damage analogue by treatment of these substrates with either endonuclease III or endonuclease IV reduced, but did not abolish, subsequent incision by the UvrABC complex, whereas introduction of a 3′ nick at an abasic site increased the incision efficiency of the UvrABC complex. These data demonstrate a convergence of base and nucleotide excision repair pathways in the removal of specific base damages.

Nucleotide excision repair represents a generalized enzymatic pathway for the removal of many types of DNA lesions, including UV-induced pyrimidine dimers and "bulky" chemical adducts such as psoralen-thymine monoadducts and cisplatin G-G intrastrand cross-links. *Escherichia coli* nucleotide excision repair is initiated by the UvrABC nuclease complex in a cascade of ordered reactions (Grossman et al., 1988; Sancar & Sancar, 1989; Van Houten, 1990). Prior to their interaction with DNA, the UvrA and UvrB subunits associate in solution in an ATP-dependent manner to form an $UvrA_2B$ complex (Orren & Sancar, 1989; Oh et al., 1989). During the damage recognition step, the $UvrA_2B$ complex probes the DNA helix for damage-induced distortions by transiently melting into the two strands during a limited helicase activity, in which ATP is absolutely required (Oh & Grossman, 1989a). This helicase activity is inhibited by the presence of a DNA lesion resulting in a stable protein-DNA complex (Husain et al., 1986; Seeberg & Steinum, 1982; Van Houten et al., 1987; Yeung et al.,

†This work was supported in part by start-up monies from the Department of Pathology UVM (B.V.H.) and grants from NIH FIRST CA50681 (B.V.H.), GM37216 (Y.W.K.), and ASC CH52672 (B.V.H.). A.S. is supported by Predoctoral Environmental Pathology Training Grant ES07122-09.

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0006-2960/90/0429-7251$02.50/0