The functional sizes of the two streptococcal hyaluronan synthases (HASs) were determined by radiation inactivation analysis of isolated membranes. The native enzymes in membranes from Group A Streptococcus pyogenes and Group C Streptococcus equisimilis HAS were compared with the recombinant proteins expressed in Escherichia coli membranes. Based on their amino acid sequences, the masses of these four proteins as monomers are approximately 48 kDa. In all cases, loss of enzyme activity was a single exponential function with increasing radiation dose. The functional sizes calculated from these data were identical for the four HASs at 64 kDa. In contrast, the sizes of the proteins estimated by the loss of antibody reactivity on Western blots were essentially identical at 41 kDa for the four HAS species, consistently lower than the functional size by 23 kDa. Matrix-assisted laser desorption time of flight mass spectrometry analysis of purified S. pyogenes HAS-H₆ and S. equisimilis HAS-H₂ gave masses that differed by less than 0.07% from the predicted monomer sizes, which confirms that neither protein is posttranslationally modified or covalently attached to another protein. Ongoing studies indicate that the purified HAS enzymes require cardiolipin (CL) for maximal activity and stability. When irradiated membranes were detergent solubilized and the extracts were incubated with exogenous CL, the resulting membranes had a larger size of the functional HAS enzyme, compared with the HAS monomer, is due, therefore, to CL molecules. We propose that the active streptococcal HA synthases are monomers in complex with 16 CL molecules.

Hyaluronan (HA) is a linear heteropolysaccharide composed of repeating disaccharide N-acetyl-D-glucosamine-β(1→4)-D-glucuronic acid-β(1→3). This relatively simple polysaccharide is found throughout the vertebrate phyla in almost every organism. HA is an almost general component of extracellular matrices and is present in large amounts to serve specialized purposes in cartilage, synovial fluid, dermis, and the vitreous humor of the eye (1–4). This glycosaminoglycan plays critical roles during fertilization, embryogenesis, development, and differentiation. HA is also found in several bacteria, notably Streptococcus Group A and Group C strains, as an external capsule that contributes to bacterial pathogenicity (5, 6). The HA capsule helps these organisms evade the host immune response. Although HA was discovered in 1934 (7), the enzyme responsible for HA biosynthesis was not identified until the Group A HAS gene was cloned in 1993 (8).

The HA synthases have at least three unique features that distinguish them from almost all of the previously characterized and cloned glycosyltransferases (9). First, although most glycosyltransferases are endoplasmic reticulum/Golgi membrane proteins, only HAS is active at the plasma membrane in both bacteria and eukaryotes (10, 11). This unusual localization for a biosynthetic enzyme is related to the second unusual feature, namely, that the product (HA) is extruded into the extracellular space, while the growing HA chain remains bound to the enzyme (12). The two substrate sugar nucleotides are presumably made and delivered to the active sites of the enzyme in the cytoplasm. HA chain release typically occurs after >20,000 monosaccharides (>4 × 10⁶ Da) have been assembled. We presently view this HA extrusion process as requiring the enzyme to form a pore- or channel-like pocket to guide the hydrophilic HA chain through the lipid bilayer. Third, the HA synthase assembles a heteropolysaccharide composed of two alternating sugars. Other polysaccharide synthases that make homopolymers with a single linkage, such as cellulose or chitin, could have a single active site. HA synthases must have the ability to bind two different substrates and to catalyze the formation of two different glycoside bonds.

The hyaluronan synthases from Group A and Group C Streptococcus are the smallest enzymes (419 and 417 amino acids, respectively) in the HA synthase family (13), which now includes at least three related mammalian enzymes that are ∼40% larger (14–19), an algal phage enzyme (20), and an even larger enzyme from Pasteurella multocida (21). The first member of this family to be cloned was spHAS. A surprising finding in the early studies of spHAS was that HA synthesis required only the single gene product, the purified HAS protein (8, 22, 23). Many investigators in the field expected that heteropolysaccharide biosynthesis would probably require at least two different enzymes, each catalyzing one of the necessary glycosyltransferase reactions. Other proteins in the bacterial HA synthesis operon are only required to produce large enough quantities of the two sugar nucleotides, UDP-GlcA and UDP-GlcNAc, so that the enzyme can create an HA capsule without
S. equisimilis (26) were cloned into pKK223-3 as described (23) and transformed into E. coli Sure cells. The pKK223-3 vector contains a strong lac promoter that can be regulated by the lac repressor and induced with isopropyl-β-D-thiogalactoside. To facilitate purification of the spHAS protein, a C-terminal fusion of 6-His residues was introduced into each construct using synthetic oligonucleotides and standard polymerase chain reaction techniques. A similar seHAS-H₄ was used only for the MALDI-TOF MS experiments. The coding sequences of clones selected for extensive study were completely sequenced to verify that no changes had occurred.

Cell Growth and Membrane Preparations—Membranes from S. pyogenes and S. equisimilis were obtained by modifications of a protoplast method (31) as reported previously (23). The membranes from E. coli were isolated by a variation of the protoplast method of Ito et al. (32). Sure cells containing the HAS-encoding plasmids were grown at 30 °C in Luria broth containing 50 mM glucose and trace elements (33) to an A₅₆₀ of 1.2. The cells were then induced with 1 mM isopropyl-β-D-thiogalactoside and grown for an additional 3 h. The cells were harvested by centrifugation at 4 °C for 30 min at 3000 × g, washed twice with PBS containing 10% glycerol, and then frozen at −80 °C. Cell pellets were thawed and resuspended to 1% of the original culture volume in 20% sucrose, 30 mM Tris-HCl, pH 8.2, 10 mM MgCl₂, 1 mM DTT, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin. Lysozyme (4 mg/ml) in 0.1 M EDTA, pH 8 (0.1% culture volume), was added, and the suspension was incubated for 40 min on ice with constant mixing. Phenylmethylsulfonyl fluoride was added to a concentration of 46 μg/ml, and the suspension was sonicated on ice three times for 30 s each at 20 watts with a microtip probe (W-380; Heat Systems Ultrasonic, Inc., Farmingdale, NY). MgCl₂, DNase, and RNase were added to final concentrations of 60 mM, 1 μg/ml, and 1 μg/ml, respectively. After 20 min on ice with constant mixing, debris was removed by centrifugation (10,000 × g, 30 min, 4 °C). The lysate was diluted 2-fold in PBS containing 10% glycerol, 1 mM DTT, and the above protease inhibitors before the membranes were harvested at 100,000 × g for 1 h. The membrane pellet was resuspended once with PBS containing 10% glycerol, 1 mM DTT, and the above protease inhibitors and centrifuged. The final pellet was stored at −80 °C.

Radiation Sample Preparation—Membranes containing native or recombinant HAS were resuspended to ~3 mg/ml protein in a cryobuffer containing 50 mM bis-[2-hydroxyethyl]iminom-tris-[hydroxy-methyl]ethanethane, pH 7, 150 mM NaCl, 20 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, and 15% (v/v) glycerol. Yeast G6PDH was added to the membrane samples to a final concentration of 60 μg/ml as an internal standard. Aliquots (0.5 ml) of the samples were frozen in dry ice in 2-ml ampoules and then sealed with an acetylene torch. Sealed samples were kept at −80 °C until they were irradiated at −135 °C with 10-MeV electrons as described by Harmon et al. (34). After the samples were irradiated, the frozen vials were opened, purged with nitrogen gas, and thawed, and the membrane suspension was used for enzyme assays. Aliquots were re-frozen for later SDS-PAGE and Western blot analysis. Additional controls also verified that HAS activity was not significantly affected by the number of freeze-thaw cycles needed to perform these inactivation studies. For example, after freezing and thawing membranes twice, the spHAS activity was 90% of the control.

Western Blotting—After SDS-PAGE, proteins were electrotransferred, using a Bio-Rad mini Transblot device, to nitrocellulose (0.1 μm; Schleicher & Schuell) at 90 V for 2 h in buffer containing 20% methanol and 0.01% SDS as described by Towbin et al. (35). The blots were briefly air dried and visualized with 0.05% of the reversible stain copper (II)-phthalocyanine-3,4,4′,4″-tetrasulfonic acid (Aldrich) in 12 mM HCl. The blots were then photographed, destained with 10 mM Tris-HCl, pH 8, 150 mM NaCl, and blocked by incubation at 4 °C overnight with 5% (w/v) BSA in the same buffer with 0.01% Tween 20 and 0.05% sodium azide. This latter buffer was used for the blocking, washing, and antibody incubations. The blots were incubated with a specific anti-peptide polyclonal rabbit IgG (directed against the sequence E₁₇₁₃₅DADVFLTVDSDT common to both HAS enzymes; (23, 26)) for 1 h and washed five times at 5 min each. The blots were then incubated with anti-rabbit IgG conjugated to alkaline phosphate for 1 h, washed as above, and developed using p-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate p-toluene salt (Bio-Rad) to visualize the bound primary antibody.

HAS Activity—Samples were assayed for HAS activity in 100 μl of 50 mM Tris-HCl, pH 7, containing 20 mM MgCl₂, 1 mM DTT, 0.1 mM EDTA, 0.05% BSA, 15% glycerol, either 1.5 mM (for spHAS) or 1 mM (for seHAS) UDP-GlcNac, 1 mM UDP-GlcA, and 0.5 μl of the 14C-UDP-GlcNAc (267 μCi/mmol; ICN, Costa Mesa, CA). To initiate the enzymatic reaction, a constant amount of membrane protein (in the range of 2–3 μg) was added to the reaction mixture. After a predetermined time, the reaction was terminated, and the reaction mixture was added to 200 μl of ice-cold 20% trichloroacetic acid (TCA) and incubated for 15 min on ice. After centrifugation, the supernatant was removed, and the pellets were washed three times with 20% TCA. The pellets were then dissolved in 0.1 M NaOH, and the concentrations of 14C-UDP-GlcNAc were measured by liquid scintillation counting. The initial activity was determined at 0 time (0 min) and used as 100% activity. The remaining activity was determined at various times, and the data were plotted on a log-log graph to determine the half-life of the assay. The half-life was then calculated by least squares analysis of the data. The initial activity was used as the concentration of substrate, and the remaining activity was used as the concentration of product. The assay was performed in triplicate, and the error bars represent the standard deviation of the triplicate measurement.

The radiation inactivation studies indicate that the surviving HAS molecules incorporate their substrates into HA in a normal manner. The goal of the present study was to determine whether the streptococcal HAS proteins function as monomers or oligomers during HA biosynthesis. Radiation inactivation analysis is a powerful and general method to determine the functional size (molecular mass) of proteins (27). A single radiation “hit” by high-energy electrons destroys the entire polypeptide, yielding multiple fragments. Because the probability of destroying a particular macromolecule is proportional to the radiation dose and the size (mass) of the target, one can calculate the mass from the relationship between the radiation dose and the extent of inactivation. The method has been used to characterize a variety of membrane receptors, transporters, and enzymes (28–30) and has the advantage that it can be applied to membranes in the absence of detergents. Therefore, the functional size of a membrane protein in its natural environment can be assessed.

In this study, we have used radiation inactivation of streptococcal membranes and mass spectrometry analysis of purified HAS to determine the size of the active HA-synthesizing species. In all cases the results support the conclusion that the active enzyme is a monomer, rather than a dimer or oligomer, of the HAS protein in complex with CL.
TABLE I

| Substrate   | Native spHAS | Recombinant spHAS-H6 | Native seHAS | Recombinant seHAS |
|-------------|--------------|----------------------|--------------|-------------------|
|             | 0 Mrad       | 24 Mrad              | 0 Mrad       | 24 Mrad           |
| K_m (μM)    | 36 ± 5       | 37 ± 2               | 39 ± 2       | 37 ± 3            |
|             | 72 ± 9       | 70 ± 6               | 73 ± 6       | 69 ± 9            |
| UDP-GlcNAc  | 151 ± 2      | 153 ± 4              | 156 ± 9      | 150 ± 1           |
| K_m (μM)    | 70 ± 7       | 72 ± 2               | 71 ± 7       | 74 ± 13           |

\[ V_{max} = \frac{V}{A_{1/2}} = kD \]

FIG. 2. Radiation inactivation of native and recombinant spHAS. Membrane samples were prepared, irradiated, and analyzed as described under "Experimental Procedures." Both native spHAS (C, dashed line) and recombinant spHAS (●, solid line) gave a single exponential inactivation curve as a function of radiation dose.

added, and the mixtures were gently mixed using a micromixer (Taiotec Inst. USA, Inc., San Jose, CA) at 30 °C. Samples were taken from 2 to 60 min, and the reactions were terminated by the addition of SDS to a final concentration of 2% (w/v). Incorporation of [14C]GlcA into high molecular weight HA product was measured (23) by descending paper chromatography using Whatman No. 3MM, developed in 1M ammonia, pH 11.7. The plate was dried and the spot was then allowed to air dry. MS analysis was performed by the MALDI-TOF mass spectrometer used was a Voyager Elite (Perceptive Biosystems, Framingham, MA) equipped with a Nd:YAG laser (337 nm). Samples were analyzed in the linear, positive mode with a delayed extraction of 250 ns and were subjected to a 25-kV accelerating voltage. All mass determinations were based on external and/or internal calibrations using two standard proteins: horse apomyoglobin and BSA, which have molecular masses of 16,851 and 66,432 Da, respectively.

**RESULTS**

Because both the spHAS and seHAS genes have been cloned, we were able to examine the recombinant enzymes expressed in *E. coli* membranes, as well as the native proteins in streptococcal membranes. In this study we use the term native to refer to the wild-type HAS protein expressed in *Streptococcus* membranes; the naturally occurring situation. After radiation of membrane samples, the activity of HAS decreased, but the residual enzyme kinetics remained linear (Fig. 1). The validity of interpreting radiation inactivation data depends on the normal kinetic behavior of surviving enzyme molecules in the irradiated membrane samples. To apply the simplest theory of radiation inactivation, it is necessary to verify that the residual enzyme activity is not altered in its kinetic properties, except for a reduced *V* \(_{max}\). In addition to retaining linearity (Fig. 1), all of the irradiated HAS membrane samples demonstrated essentially unchanged *K* \(_m\) values for the UDP-sugar substrates (Table I), indicating that the surviving HAS molecules were able to function normally. The decrease in *V* \(_{max}\) with radiation dose was, therefore, suitable for a simple target analysis of enzyme inactivation.

The activity of spHAS in either streptococcal or *E. coli* membranes demonstrated identical single exponential decay curves when exposed to increasing radiation doses (Fig. 2). The results from a number of experiments are summarized in Table II.

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Based on inactivation of enzyme activity in three separate experiments, the functional size of the native spHAS ranged from 48 to 64 kDa. The average target size was 58 ± 9 kDa. The target size of the recombinant spHAS in E. coli membranes ranged from 60 to 80 kDa in seven experiments. The average target size was 67 ± 8 kDa.

The native spHAS protein contains 419 amino acids and has a calculated mass of 47,774 Da (23). The calculated mass for spHAS-H6 is 1.9% greater at 48,677 Da. For purposes of affinity purification, the recombinant spHAS has a C-terminal His6 tail that does not affect HA synthase activity. The radiation inactivation results indicate that the native and recombinant spHAS proteins have essentially identical functional sizes that correspond more closely to a monomer rather than a dimer of HAS (which has a predicted size of ~97 kDa). Although the average calculated target size of ~63 kDa for the active spHAS is ~30% larger than expected for a monomer, it is inconsistent with the active species being a homodimer. The larger-than-expected size for active spHAS indicates that additional mass of ~15 kDa is required for enzyme activity. This extra mass could be even greater (by ~8 kDa) based on the observed HAS size versus the calculated size (see below).

The recently cloned seHAS (26) was also examined by radiation inactivation, using the recombinant seHAS expressed in E. coli membranes and the native enzyme in Group C streptococcal membranes (Fig. 3 and Table III). The Group C HAS contains 417 amino acids (47,778 Da) and is 70% identical in primary sequence to the Group A enzyme. In four experiments, the native seHAS functional size ranged from 47 to 69 kDa, with an average of 62 ± 10 kDa. The recombinant seHAS indicated a target size of 72 ± 8 kDa in four experiments. Again, the functional sizes of the native or recombinant seHAS enzymes (average of ~67 kDa) were not significantly different from each other or from the sizes observed for the spHAS enzymes. These data indicate that the functional unit of both streptococcal HA synthases contains a single copy, rather than multiple copies, of the HAS protein. As in the case of spHAS, the observed functional size of seHAS is ~19 kDa larger than the predicted size (or ~25 kDa larger than the size observed by radiation analysis of Western blots).

**TABLE II**

Radiation inactivation analyses of Group A spHAS

| Exp. | Recombinant spHAS-H6 (E.c.) | Native spHAS (S.p.) |
|------|-----------------------------|---------------------|
|      | Activity Western            | Activity Western G6PDH |
|      | G6PDH                       |                     |
| 1    | 64 ± 9 (n = 10) 32           | 48 ± 5 (n = 7) 44 (n = 2) 104 |
| 2    | 60 ± 3 (n = 9) 57 (n = 2)    | 64 (n = 2) 41 (n = 2) 119 |
| 3    | 61 ± 2 (n = 8) 36 (n = 2)    | 62 (n = 2) 40 (n = 2) 119 |
| 4    | 66 (n = 2) 45 (n = 2)        |                     |
| 5    | 64 (n = 2) 45 (n = 2)        |                     |
| 6    | 77 (n = 2) 28 (n = 2)        |                     |
| 7    | 80 (n = 2) 25 (n = 2)        |                     |
| 129 ± 20 (n = 6) 67 ± 8 (n = 35) 38 ± 11 (n = 13) | 58 ± 9 (n = 11) 42 ± 2 (n = 6) 114 ± 9 (n = 3) |

**TABLE III**

Radiation inactivation analyses of Group C seHAS

| Exp. | Recombinant seHAS (E.c.) | Native seHAS (S.e.) |
|------|--------------------------|---------------------|
|      | Activity Western G6PDH    | Activity Western G6PDH |
|      | kDa                      | kDa                 |
| 1    | 125 78 (n = 2) 46 (n = 2) | 47 ± 10 (n = 9) 27 (n = 1) |
| 2    | 126 78 (n = 2) 31 (n = 2) | 69 ± 11 (n = 8) 44 (n = 2) 106 |
| 3    | 119 66 (n = 2) 47 (n = 2) | 66 (n = 2) 57 (n = 2) 117 |
| 4    | 120 64 (n = 2) 47 (n = 2) | 66 (n = 2) 36 (n = 2) 117 |
| 123 ± 4 (n = 4) 72 ± 8 (n = 8) 43 ± 8 (n = 8) | 62 ± 10 (n = 21) 41 ± 13 (n = 7) 110 ± 12 (n = 7) |
Additional insight into the molecular organization of the HAS protein, independent of its enzyme activity, can be determined by analysis of HAS protein loss, based on SDS-PAGE analysis, with increasing radiation dose (Fig. 4). The streptococcal HAS proteins migrate anomalously fast during SDS-PAGE, with an apparent $M_r$ of 42,000, rather than the predicted $M_r$ of 48,600 (8, 23, 26). Western blot analysis of irradiated E. coli membranes containing spHAS-H$_6$ (Fig. 4A) shows a progressive loss of staining of the HAS protein as the dose increases. Calculation of the HAS target size in this experiment is not related to the position at which the protein migrates during SDS-PAGE but rather to the loss of antibody-reactive material at this position as a function of radiation dose. The intensity of the surviving monomer protein bands for native and recombinant spHAS or seHAS follow the same simple exponential function versus radiation dose seen in the above inactivation experiments (Fig. 5). However, in each case the target size obtained from analysis of the Western blot was consistently smaller than the target size based on enzyme activity. The spHAS target sizes based on this analysis of HAS protein destruction were 42 ± 2 and 38 ± 11 kDa for the native (Fig. 5A) and recombinant (Fig. 5B) enzymes, respectively (Table II). Similarly, the native (Fig. 5C) and recombinant (Fig. 5D) seHAS target sizes were 41 ± 13 and 43 ± 8 kDa, respectively (Table III), indicating that neither the spHAS nor seHAS protein exists physically in membranes as a covalently linked oligomer. This result is consistent with the observation that the streptococcal HASs are not disulfide bonded to themselves or other proteins (8, 23, 26).

Because the recombinant seHAS and spHAS proteins constitute 5–8% of the total membrane protein in E. coli (26), the enzyme band is very apparent in Coomassie Blue-stained gels after SDS-PAGE (Fig. 4B). The control using cells transformed with vector alone shows essentially no protein staining at the same position (26). The total HAS protein clearly decreases with increasing radiation dose, confirming that it is destroyed. Unfortunately, HAS protein loss cannot be followed accurately in such gels after irradiation of samples, because of the changing background in each lane (at each dose) as radiation-induced protein degradation proceeds.

The larger-than-monomer size of the functional enzyme could reflect the addition of one or more relatively small molecules to HAS to account for 23 kDa. Radiation inactivation analysis only detects the polypeptide portion of macromolecules and, for example, not covalently bound carbohydrate or very tightly bound lipid, unless those components are required for the function being assessed (27, 26). Disulfide-bonded proteins behave as one larger target (27). To assess the possibility that HAS contains additional covalently attached components of any nature, we used MALDI-TOF mass spectrometry to analyze affinity-purified spHAS-H$_6$ and seHAS-H$_6$ (Fig. 6). The purified enzymes are 98% homogeneous and very active. Consistent with the predicted mass for spHAS-H$_6$ (48,677 Da), in three experiments we observed a substantial peak at 48,680 ± 13 Da. This mass was very reproducible among different enzyme preparations (Table IV).

Essentially identical results were obtained for the purified seHAS-H$_6$ (predicted mass, 48,600). The mean value observed in four experiments was 48,567 ± 21 (Table IV). Additionally, no species were detected at 60 kDa in five independent preparations of either seHAS-H$_6$ or spHAS-H$_6$ (Fig. 6). As expected, there was also no indication of a substantial dimeric species, which would likely require disulfide bonding to stay associated after laser excitation and during MALDI-TOF analysis. Only minor peaks were observed at the HAS dimer and trimer positions, but small amounts of artifactual dimer and trimer were

![Fig. 4. SDS-PAGE analysis of spHAS-H$_6$ in irradiated membranes. A, Western blot analysis of irradiated membranes containing recombinant spHAS was performed as described under "Experimental Procedures" using 30 µg of protein/lane. The Western analysis shows a progressive loss of intact HAS protein (arrow) in the irradiated samples compared with the nonirradiated sample. Smaller fragments of HAS are evident at higher doses. B, Coomassie-stained gel. Because spHAS and seHAS are ~5–8% of the total membrane protein, the enzyme (arrow) is visualized with Coomassie Blue after SDS-PAGE. The Coomassie Blue staining shows a progressive decrease of HAS protein as the radiation dose increases. There is also a coincident decrease in larger species as well as an increase in the number of small molecular weight (damaged) proteins. Lanes in A and B (doses in megarads): 1, 0; 2, 6; 3, 12; 4, 24; 5, 36; 6, 48; 7, 60; 8, 72; 9, 96; 10, 120.](image-url)
also observed for the monomeric internal protein standards (BSA and myoglobin) used to calibrate the instrument. Although the failure to detect a species must be interpreted cautiously, the results support the conclusion that the active HAS enzyme contains a single 48-kDa HAS protein, without covalent modifications.

A previous report showed that spHAS could be solubilized from native membranes and reconstituted only if CL were present (41). Consistent with this, we find that purified spHAS-H₆ or seHAS-H₆ has very low activity and is less stable when CL is depleted. Addition of exogenous CL to the lipid-free, purified protein increases enzyme activity at least 10-fold. Furthermore, successful and reproducible purification of active, stable enzyme is only consistently achieved when CL is

FIG. 5. Analysis of radiation-induced HAS protein loss. Irradiated membranes containing native spHAS (A), recombinant spHAS-H₆ (B), native seHAS (C), or recombinant seHAS (D) were analyzed by SDS-PAGE, Western blotting, and densitometry as described under “Experimental Procedures.” The dashed line in each panel represents the regression line for the inactivation of enzyme activity as determined in Figs. 2 and 3. In all four cases, the loss of HAS protein is less sensitive to irradiation (i.e., has a shallower slope) than HAS enzyme inactivation, indicating that the active enzymes are larger than a HAS protein monomer.
included during the extraction and storage steps. Based on these results, we hypothesized that CL could account for the additional mass of ~23 kDa apparently required for enzyme activity, as revealed by the radiation inactivation analysis. To test this hypothesis, irradiated membranes containing spHAS-H6 were preincubated with or without E. coli CL before determining HAS enzyme activity.

When nonirradiated membranes were preincubated with CL (Fig. 7), the HAS activity increased significantly (~30%). Surprisingly, this increased HAS activity was not observed when CL was added to irradiated membranes. However, when the membranes were first solubilized using the detergent DDM, a very large stimulation of enzyme activity by CL (2.9-6.0-fold) was then observed (Fig. 8). The stimulation of HAS activity by CL occurred with both solubilized nonirradiated and irradiated membranes. This effect of CL means that, at a given radiation dose, more residual spHAS enzyme activity remains than revealed by assay of the intact membranes. Consequently, the target size for HAS activity in the CL-supplemented extracts (Fig. 9) was smaller than that observed in the above experiments (Fig. 2).

The HAS target size in the presence of CL was reduced by 29 ± 5% (Table V). In the presence of CL the active HAS size was 57 ± 4 kDa, compared with 80 ± 8 kDa in the absence of added CL. Another important finding also shown in Table V is that extraction of the HAS protein, from irradiated membranes into the detergent DDM, had no effect on the target size of active HAS. Studies on the detergent-solubilized protein

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**TABLE IV**

| SpHAS-H6 | SeHAS-H6 |
|----------|----------|
| Exp. | Mass | Exp. | Mass |
| 1 | 48,680 ± 2 (n = 2) | 1 | 48,573 ± 12 (n = 7) |
| 2 | 48,679 ± 16 (n = 6) | 2 | 48,589 ± 15 (n = 6) |
| 3 | 48,689 ± 11 (n = 8) | 3 | 48,556 ± 20 (n = 4) |
| 4 | 48,674 ± 10 (n = 10) | 4 | 48,554 ± 13 (n = 8) |
| Mean | 48,680 ± 13 (n = 26) | 48,567 ± 21 (n = 25) |
| Predicted | 48,677 | 48,600 |

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**Fig. 6.** MALDI-TOF Mass Spectrometric analysis of affinity purified sp/seHAS-H6. The linear, positive-ion spectrum of recombinant spHAS-H6 (A) and seHAS-H6 (B) shows molecular (MH)\(^+\) ions at m/z 48,678 and 48,551, respectively, and doubly charged (MH)\(^{2+}\) ions at m/z 24,346 and 24,280, respectively. The calibrated spectra are the average of 95 (A) and 65 (B) scans. The partially resolved peaks at m/z 48,883 (A) and 48,754 (B) are likely matrix ion adducts to the proteins (61). Sinapinic acid (M, 224) can undergo a loss of –OH, during a dehydration reaction to form an adduct, resulting in the addition of 207 mass units to the protein.
should, therefore, be an appropriate model for understanding how the streptococcal HAS enzymes would function in a biological membrane.

The apparent mass difference of ~23 kDa for active spHAS with or without CL corresponds to the difference noted above between the active spHAS species and the spHAS protein. Similar results were obtained with seHAS membranes, although the CL stimulation of enzyme activity after extraction from irradiated membranes was less than that observed for spHAS.

DISCUSSION

The two streptococcal HA synthases are the smallest members of the HAS family and likely represent the best opportunity to elucidate the mechanism and details of HA biosynthesis (13) by this enzyme family. The present study was undertaken to determine whether the multiple functions needed for HA biosynthesis are mediated by a single HAS monomer. Previous studies have provided genetic and biochemical evidence that no other protein except HAS is required to make an active HAS (8, 22, 23, 26). The radiation inactivation data show that all four HAS preparations studied behave identically. The two native HAS enzymes in streptococcal membranes or the two recombinant HAS proteins in *E. coli* membranes gave the same results. All these data are consistent with a simple single exponential inactivation, indicating that no unusual or complicated species (e.g. inhibitor or activator proteins) are present.

The active seHAS and spHAS species are ~65 kDa, which is ~17 kDa larger than the calculated mass of the proteins and ~24 kDa larger than the HAS size determined by radiation inactivation (~41 kDa). These differences between protein size and size of the active species were consistent and reproducible, from which we conclude that the active HAS must contain more than the HAS protein. Because this additional mass of whatever else is needed for activity is <50% the size of the HAS protein, the active HAS species cannot be a dimer or homooligomer of the HAS protein. Only one HAS protein is required to assemble an active HA synthase. Furthermore, based on the protein migration in SDS-PAGE, the protein size by radiation inactivation, and the accurate mass determination of the purified HAS using MALDI-TOF MS, the active enzyme is not covalently attached to any other proteins. The active spHAS-H<sub>6</sub> and seHAS-H<sub>6</sub> also do not contain any posttranslational modifications such as phosphoryl or fatty acyl groups.

Radiation inactivation analysis reveals the total mass of all components required for the activity being measured (27, 36). In the case of an enzyme requiring two noncovalently associated subunits, the target size would be the sum of both subunit masses, provided that subunit rearrangements do not occur subsequently to create new, undamaged heterodimers. In fact, subunit exchange does not seem to occur. For example, Boyer and Kempner (42) found no rearrangement of glutathione S-transferase after irradiation. Other studies have also shown that a variety of irradiated proteins do not dissociate, precluding any exchange of subunits (43, 44). Similar reasoning would apply to a HAS protein whose activity required the noncovalent association of other molecules (cofactors). The target size of the active species would be larger than a HAS monomer if the enzyme were inactivated by a radiation event that destroyed the noncovalently associated components. Such inactivation without destruction of the HAS protein would only occur if the damaged cofactor could not be replaced (and the enzyme, therefore, reactivated) by exchange with other cofactor molecules in the irradiated membranes.

In ongoing experiments, we find that purified seHAS-H<sub>6</sub> and spHAS-H<sub>6</sub> require CL for both maximum activity and stability. Because CL is a prevalent phospholipid in *E. coli* and other bacterial membranes (45), the overexpressed HAS protein can associate with this CL and be activated. The finding (Fig. 7) that exogenous CL enhances HAS activity in *E. coli* membranes indicates that the ratio of CL to HAS protein is not high enough to give maximum activity of the overexpressed HAS, which constitutes ~5–8% of the total membrane protein. We assume in this experiment that the added CL quickly intercalates into the natural membranes and is able to interact with, and activate, HAS enzymes that are either partially or completely inactive.

Surprisingly, the irradiated membranes did not show increased HAS activity when CL was added, which suggests that damaged CL may remain associated with the HAS protein. Perhaps radiation-induced CL fragments do not exchange efficiently with exogenous CL in the membranes. Alternatively,
the irradiated membranes may have an altered ability to take up the added CL, or the rates of CL diffusion within these membranes may be greatly reduced compared with nonirradiated membranes. In any case, the exogenous CL would not be able to rescue HAS activity by replacing damaged CL associated with the enzyme. Extraction of HAS from irradiated membranes, however, might circumvent several of these possibilities and facilitate the interactions of inactive HAS with exogenous CL.

The results in Figs. 7–9 are consistent with the above scenario and demonstrate that the apparent size of the active HAS is decreased by ~23 kDa when CL is not limiting. Because the mass of CL is ~1400–1500 Da depending on the fatty acid composition, we conclude that a single HAS protein must interact with ~16 CL molecules to be active. Because the activation of purified HAS by CL only occurs above a critical concentration of ~0.5 mM CL,2 we believe that a collection of CL molecules is required to activate the enzyme in a cooperative manner. Loss of only one CL from this complex by radiation damage apparently can cause inactivation of the enzyme.

Other enzymes have been found to have essentially absolute requirements for particular phospholipids including CL (45). Cardiolipins are abundant in mitochondria and play critical roles in oxidative phosphorylation by interacting with a variety of membrane proteins (46). The ADP/ATP carrier has an absolute requirement for CL to mediate nucleotide translocation (47). CL also modulates the secondary structure of cytochrome oxidase (48) and the activity of cytochrome C oxidase (49). The latter enzyme was stimulated by exogenous CL but not other phospholipids, a result similar to our present finding with streptococcal HAS. Other enzymes dependent on CL for their activity include cytochrome P450SSC (50), nitric oxide reductase (51), protein kinase N (52), and protein kinase Ce (53). The phospholipid-binding serum protein β2-glycoprotein I, which has anticoagulant properties, specifically binds CL at a highly positively charged heptapeptide sequence, KKNEKK (54). Similarly, L-selectin, but not E-selectin, is suggested to bind specifically to CL at a related sequence, KKKKEK (55).

Only two other phospholipid-dependent enzymes have been analyzed by radiation inactivation. Erythrocyte acetylcholinesterase, which is modulated kinetically by association with 33 molecules of CL (56), showed no evidence that CL was part of the enzyme target size. d-β-Hydroxybutyrate dehydrogenase is a lecithin-dependent mitochondrial enzyme. Radiation inactivation analysis (57) showed that inactivation of this enzyme was not attributable to phospholipid destruction, nor did lecithin activate the enzyme by regulating oligomerization. The results from radiation studies of both these other enzymes indicate that the phospholipid required for enzyme activity was not seen in the target size, because the damaged phospholipids were readily replaced by undamaged lipid molecules in these enzyme preparations. In our case, the radiation-damaged CL associated with HAS does not appear to exchange until the protein is detergent solubilized.

Ongoing studies on the topology of spHAS (58) indicate that the model proposed initially is essentially correct (8, 13, 22, 26). The protein appears to have four transmembrane domains and one or more amphiphilic helices that may be partially imbedded in the membranes as reentrant loops (59). Translocation of an HA chain across the bilayer is difficult to envision for such a small protein. Typical membrane transporters for small molecules such as glucose or amino acids usually contain >10 transmembrane domains to create pores (60). We propose that a collection of CL molecules may be critical to enable the HAS protein to create or maintain a pore-like structure with a hydrophilic interior that interacts with HA and a hydrophobic exterior that interacts with the lipid bilayer. Further studies will of course be necessary to verify this intriguing possibility.

In summary, the results support the conclusion that both the Group A and Group C streptococcal HA synthases form a functionally active enzyme complex that contains one HAS protein and ~16 CL molecules.

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