Physical Characterization of a Reactivatable Liposome-Bound Rhodanese Folding Intermediate†

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ABSTRACT: Recently, we described the formation of a complex between liposomes and the unfolded protein rhodanese (thiosulfate:cyanide sulfurtransferase, EC 2.8.1.1), which could be liberated and efficiently reactivated after treatment of the complex with detergents [Zardeneta, G., & Horowitz, P. M. (1992) *Eur. J. Biochem. 210,* 831–837]. Previous data suggested that liposome-bound rhodanese was in the form of a folding intermediate. We have characterized in greater detail the nature of the conformation of the bound rhodanese. Physical characterization of the bound rhodanese intermediate was carried out using proteolysis, fluorescence studies with 1,8-anilinonapthalene-8-sulfonic acid, a probe for hydrophobic site exposure, intrinsic fluorescence to determine tryptophan accessibility using the quenchers acrylamide and iodide, and circular dichroism to detect extent of secondary structure. These studies show that the rhodanese intermediates bound to either cardiolipin or phosphatidylserine liposomes are not identical, the former being in a less compact conformation yet having more secondary structure than the latter, an observation which may explain why the reactivation of the former intermediate is more effective. Finally, turbidimetric and proteolytic studies raise the possibility that each rhodanese intermediate binds to several liposomes. This finding suggests that a possible reason for the differential reactivation yields obtained may be due to the fact that unfolded rhodanese has more binding sites for cardiolipin than for phosphatidylserine liposomes. A greater number of binding sites would result in better anchoring of rhodanese's interactive surfaces and thus reduce the likelihood of misfolding.

The processes which lead to the correct folding of a denatured or newly synthesized protein are incompletely understood [for reviews, see Ptitsyn (1987) and Kim and Baldwin (1982)]. One model suggests that folding begins with the discrete formation of secondary structure at defined segments of the unfolded protein and then proceeds from that point (Anfinsen, 1972), while a second model proposes independent folding of different segments of the protein occurring simultaneously (Ptitsyn & Rashin, 1973). *In vitro,* an unfolded protein can either fold correctly, misfold, or form kinetically trapped aggregates. Generally, a significantly high percentage of proteins result in nonnative, inactive misfolded or aggregated conformers (Mitraki & King, 1989; Fischer & Schmid, 1990). Similarly, *in vivo,* the formation of active proteins from newly synthesized proteins involves kinetic competition between the reactions leading to different end products. In recent years, molecular chaperones, which aid in the formation of native proteins, have been identified and preliminarily characterized [for reviews see Georgopoulos (1992) and Gething and Sambrook (1992)]. Beckmann *et al.* (1990) have shown the need for the chaperone hsp70 in the prevention of misfolding of nascent polypeptides, while Nelson *et al.* (1992) show that an isotype of hsp70 binds to nascent polypeptides. We have shown that the chaperonins GroEL and GroES greatly enhance the production of active rhodanese protein in *in vitro* transcription/translation reactions (Tsalkova *et al.*, 1993).

It has become clear that an understanding of the conformation of protein folding intermediates is important in order to decipher the sequential events involved in the acquisition of a protein's tertiary structure. This knowledge may aid in the design and construction of medically important recombinant proteins that would be active when expressed in the host cell (*e.g.,* bacteria) and avoid the formation of inclusion bodies, *e.g.,* insulin (Williams *et al.,* 1982). Several processes have been deemed critical in the folding process; these include the prevention of hydrophobic or electrostatic interactions and disulfide bond formation which result in protein misfolding.

The folding conditions for rhodanese, a 33-kDa sulfurtransferase protein (EC 2.8.1.1), have been extensively studied. This enzyme is synthesized in the cytoplasm but is imported into the mitochondria and eventually localizes to the mitochondrial matrix (Boggaram *et al.,* 1985). Rhodanese has four cysteine residues which are reduced in the native enzyme, it is monomeric (Ploegman *et al.,* 1978), and its only posttranslational modification is the removal of the initiating methionine (Miller *et al.,* 1991), qualities that make it an excellent model for folding studies. Reducing agents, such as β-mercaptoethanol (β-ME),[1] and native rhodanese's (R_n) substrate, thiosulfate, are necessary to accomplish refolding (Tandon & Horowitz, 1989). In combination with these components, efficient refolding of denatured rhodanese (R_d) can be achieved by the use of detergents such as lauryl maltoside (LM) (Tandon & Horowitz, 1989), chaperonins

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[1] Abbreviations: LM, lauryl maltoside (dodecyl β-D-maltoside); PS, phosphatidylserine; CL, cardiolipin; EDTA, ethylenediaminetetraacetate, sodium salt; CD, circular dichroism; LUV, large unilamellar vesicles; ANS or 1,8-ANS, 1,8-anilinonapthalene-8-sulfonic acid; NATA, *N*-acetyltryptophanamide; R_d, denatured rhodanese; R_n, native rhodanese; R_i, rhodanese intermediate; β-ME, β-mercaptoethanol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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(Mendoza *et al.*, 1991; Martin *et al.*, 1991; Bochkareva *et al.*, 1992), mixed micelles containing LM and the mitochondrial lipid cardiolipin (CL) (Zardeneta & Horowitz, 1992a), or mediated by initially binding of R_d to phosphatidylserine (PS) or CL large unilamellar vesicles (LUV) (Zardeneta & Horowitz, 1992c). Rhodanese folding intermediates bound to GroEL have been characterized (Mendoza *et al.*, 1992). Additionally, we have characterized rhodanese intermediates that were formed by equilibrating the protein at various urea concentrations (Horowitz & Criscimagna, 1990).

Rhodanese, complexed to either CL or PS LUVs, was previously only minimally characterized (Zardeneta & Horowitz, 1992c). The intrinsic fluorescence λ_{max} of rhodanese bound to either anionic liposome was ~342 nm, which was consistent with it being a rhodanese folding intermediate (R_i). However, the two liposome-bound species were not identical in conformation, since CL-bound R_i was highly reactivatable (~60%), while the PS-bound R_i could only be reactivated to ~9%. Further, fewer CL-LUVs than PS-LUVs were required to bind a given amount of unfolded rhodanese. These LUV-bound species permit the facile characterization of rhodanese intermediates since these are held in quasistable conformations when sequestered by liposomes.

The study of protein folding intermediates is of interest not only in the study of protein folding *per se* but also because it aids in the understanding of other biochemical events, such as protein translocation across biological membranes. Imported proteins need to have a translocation competent conformation, *i.e.*, be partially folded (Chen & Douglas, 1987; Kumamoto, 1991) for effective translocation across membranes. We have shown that both GroEL (Mendoza *et al.*, 1992) and CL-LUV (Zardeneta & Horowitz, 1992c) can sequester R_d but not R_n in partially folded conformations. Others have shown that the chaperone hsp70 transports proteins in a partially folded form to the outer mitochondrial membrane (Deshaies *et al.*, 1988). There is accumulating evidence that CL, which in eukaryotes is specifically found in mitochondrial membranes, may be a target for proteins to be imported (Ou *et al.*, 1988; Eilers *et al.*, 1989; Zardeneta & Horowitz, 1992b,c) and thus perhaps have a role during protein translocation.

In the present work, we have studied the conformational similarities and differences of the rhodanese intermediates bound to either PS or CL liposomes using fluorescence techniques, circular dichroism, protease susceptibility, turbidimetry, analysis of cysteine's sulfhydryl groups accessibility, and protein aggregation via cysteine disulfide bond formation using DTNB and SDS–PAGE. Several other works have shown that, in the presence of lipid environments, proteins (Malikayil *et al.*, 1992; de Hong *et al.*, 1992; Weaver *et al.*, 1992) and protein signal sequences (Roise *et al.*, 1986; Frey & Tamm, 1990; De Kroon *et al.*, 1990) undergo large conformational changes. Here, we show that the conformation of R_i when bound to CL is more near-native than when bound to PS. Data presented here imply that each rhodanese molecule binds to more than one liposome, resulting in the formation of a turbid solution where liposomes are cross-linked to each other via rhodanese. The findings suggest that the most important determinant in the reactivability of liposome-bound intermediates is the number of binding sites that the protein has for those liposomes and/or the positions in the protein molecule of the binding sites, *i.e.*, whether they are in areas which contain highly interactive residues that cause unfolded or partially folded proteins to undergo intra- or intermolecular aggregation .

MATERIALS AND METHODS

Materials

Lauryl maltoside (dodecyl β-D-maltoside) was purchased from Anatrace (Maumee, OH); cardiolipin (beef heart) and phosphatidylserine (brain) were obtained from Avanti Polar Lipids (Alabaster Al); subtilisin was purchased from Boehringer Mannheim (W. Germany); and 1,8-ANS (1-anilinonapthalene-8-sulfonic acid) was from Molecular Probes (Junction City, OR). DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] was obtained from Sigma. All other chemicals were of reagent grade.

Methods

Rhodanese Preparation and Quantification. Bovine liver rhodanese was prepared as previously described (Horowitz, 1978), stored at −70 °C in the presence of 1.8 M ammonium sulfate and 1 mM thiosulfate, and subsequently purified to homogeneity by a series of ammonium sulfate and phosphate buffer washes. Enzyme was quantified spectrophotometrically using an extinction coefficient of 57 750 $M^{-1}cm^{-1}$, at 280 nm, and an M_r ~33 000.

Liposome Preparation. Large unilamellar vesicles (LUVs) composed of either 100% PS or 100% CL were prepared in buffer A [20 mM Pipes (pH 7.2), 1 mM EDTA, and 100 mM NaCl] at a final concentration of 5 mg/mL, as detailed previously (Zardeneta & Horowitz, 1992c), using a LiposoFast extrusion device (MM Developments, Ottawa, Canada) as detailed by the manufacturer (MacDonald *et al.*, 1991). Formed LUVs were stored at room temperature under nitrogen and used within 24 h of preparation. Some LUVs were prepared in the presence of fluorophore as indicated under Results.

Liposome–Rhodanese Complex Formation. The liposome–rhodanese complex was formed by incubating 40 μg of denatured rhodanese (R_d) with 1 mg of either PS or CL LUVs in buffer A in a total volume of 1 mL, for 1 h at 25 °C. R_d was prepared by incubating native rhodanese (R_n), at 1 mg/mL, in a solution of 9 M urea and 14 mM β-ME for 30 min at 25 °C. In the experiments indicated, β-ME was omitted from the denaturing buffer.

Protease Digestion of Rhodanese and Protein–Liposome Complex. The rhodanese/LUV complex was prepared as described above and then proteolyzed. The reaction solution contained 19.2 μg of R_d, 480 μg of either PS or CL LUVs, and 0.2 μg of subtilisin in buffer A, in a total volume of 500 μL. Digestion was carried out for 30 min at 25 °C; aliquots (40 μL) were removed at various times and analyzed by SDS–PAGE. R_n was digested and analyzed in a similar manner, except that there were no LUVs in the reaction.

Fluorescence Measurements. All measurements were performed in an SLM fluorometer (model 500C) at 25 °C. For measurements of rhodanese's intrinsic fluorescence, the excitation wavelength was 295 nm, and an emission spectra from 310 to 390 nm was obtained. The technical spectra were smoothed to identify λ_{max}, and the intensity value at the maximum was used for calculations involving quenching by either iodide or acrylamide. The data were plotted according to the Stern–Volmer equation:

$$F_o/F = 1 + K_q[Q] \quad (1)$$

where F_o is the sample's initial fluorescence intensity in the absence of quencher, and F is the sample's fluorescence intensity in the presence of quencher at a given molar concentration [Q]. K_q was calculated by linear regression

and represents the quenching constant in M^{-1}, and the number "1" represents the y-intercept of the plot. The collisional quenching constant, K_{sv}, was determined using

$$F/F_o = (1 + K_{SV}[Q]) \exp(V[Q]) \quad (2)$$

where V is the static quenching constant. The K_{sv} was estimated with the aid of a nonlinear least-squares curve fitting iterative program (Eftink & Ghiron, 1981) and by using the K_q value from eq 1 as a starting point for determination of the K_{sv} value, and by setting the static quenching value, V, equal to 10% that of K_{sv}.

In experiments involving the fluorophore 1,8-ANS, samples were excited at 370 nm, and emission spectra from 400 to 600 nm were acquired. The λ_{max} as well as fluorescence intensity at this point were recorded. Spectra of appropriate buffer solutions (blanks) were subtracted from all of the above acquisitions. Additionally, the spectra were corrected for the inner filter effects due to the quenchers.

Circular Dichroism Measurements. Denatured rhodanese was complexed with either CL or PS LUVs as indicated above and diluted in buffer A at 0.05 mg/mL, and the resulting complex was analyzed in a 1-mm path length cell by CD using a JASCO J500c instrument. Spectra between 530 and 215 were obtained at room temperature. The results shown are a curve-smoothing representation of an average of three spectral acquisitions. Solutions of native rhodanese in buffer A, in the presence or absence of liposomes, were analyzed similarly. Control spectra of solutions containing all components, except the protein, were acquired, and these spectra were subtracted from the appropriate samples' spectra. These data were further processed to determine mean residue molar ellipticity. It was not possible to collect reliable data below 215 nm, due to light scattering by the LUVs.

Determination of Accessible Sulfhydryl Groups and Detection of Disulfides in Rhodanese Species. Complexes of R_i with either PS-LUV or CL-LUV were formed as above at a rhodanese concentration of 0.04 mg/mL and a lipid concentration of 1 mg/mL in a total volume of 250 μL. After 15 min of incubation, the solutions were made 3 mM in DTNB and incubated 3 min at room temperature. Then the solution was made 1% in SDS to allow accessibility of the SH groups to the probe, DTNB. Samples were then analyzed by SDS–PAGE on 15% polyacrylamide gels under either reducing or nonreducing conditions.

Polyacrylamide Gel Electrophoresis. SDS–PAGE was carried out using a Hoeffer minigel apparatus (San Francisco, CA) and using discontinuous 15% polyacrylamide gels prepared according to Laemmli (1970). Protein bands were detected by either Coomassie blue or silver stain (Merril *et al.*, 1981).

Turbidimetry. Denatured rhodanese at 0.04 mg/mL was incubated with either PS or CL LUVs at 0.45 mg/mL in buffer A in a total volume of 0.5 mL. The changes in the turbidity (light scattering) of the R_i/liposome complexes, over time, were monitored spectrophotometrically at 400 nm in a cuvette (1-cm path length), a wavelength at which neither protein nor liposome absorb light.

RESULTS

Rhodanese Complexed with Liposomes Is More Sensitive to Proteolysis Than Native Rhodanese. Rhodanese was denatured, complexed with either PS or CL LUVs, and then digested with subtilisin as outlined under Methods. As a reference, native rhodanese was digested in a similar manner.

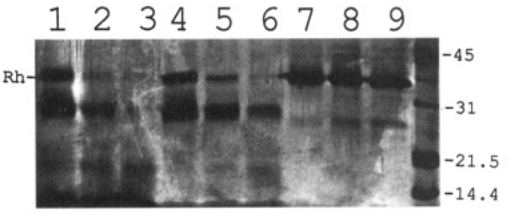

FIGURE 1: Protease digestion of liposome-bound rhodanese. The rhodanese–LUV complex was formed and digested with subtilisin as detailed under Methods. An aliquot containing 1.5 μg of rhodanese was analyzed by electrophoresis in a 15% SDS–polyacrylamide gel, followed by silver stain. Lanes 1–3 correspond to R_i/CL-LUV after 1, 5, and 15 min of digestion; lanes 4–6 correspond to R_i/PS-LUV after 1, 5, and 15 min of digestion; and lanes 7–9 correspond to R_n after 1,5, and 15 min of digestion, respectively. The molecular weights of standard proteins shown in the right lane are given. The native rhodanese migration position is indicated by "Rh".

Figure 1 shows the digestion pattern of these rhodanese species after digestion for 1, 5, and 15 min at 25 °C. The digestion was stopped by the addition of PMSF and a 40-μL aliquot (1.5 μL of protein) was analyzed by SDS–PAGE. After 15 min, under these conditions, a very small amount of R_n was digested, while the intermediate forms of rhodanese (R_i) bound to either LUV were digested to a large extent. Digestion of LUV-bound R_is produced a large fragment ($\sim$21 kDa) which was somewhat resistant to further digestion. This experiment suggests that the R_is are indeed protein folding intermediates, since their protease susceptibility, and hence their conformation, is not identical to native rhodanese.

There Are Exposed Hydrophobic Surfaces on Rhodanese When Bound to Liposomes. We used the fluorophore 1,8-ANS to determine the relative amount of exposed hydrophobic surfaces in the CL-LUV and PS-LUV complexes, as compared to each other and to R_n. ANS is not very fluorescent in aqueous solutions, but its quantum yield increases dramatically upon addition of proteins or lipids containing hydrophobic pockets or surfaces. Thus, large unilamellar vesicles used in these experiments were prepared in buffer A in the presence of 20 M ANS so that the effect of addition of protein could be clearly distinguished over basal levels of fluorescence due to lipid/fluorophore interactions. The LUV solutions containing 20 M dye inside and outside the liposomes were supplemented with buffer A and 1,8-ANS to give final concentrations of 0.25 mg/mL lipid and 20 M ANS in a total volume of 600 μL; spectra of these samples were acquired and shown in Figure 2. Subsequently, 4 μg of either R_n or R_d were added to these solutions and spectra acquired 1 min after addition of protein. Figure 2A shows that native rhodanese did not increase the quantum yield of ANS fluorescence while addition of unfolded rhodanese showed a significant fluorescence increase. Furthermore, a modest 5% increase in fluorescence was obtained 15 min after binding of R_d to CL-LUVs.

Figure 2B shows the fluorescence intensity spectra as a function of wavelength of samples containing PS-LUV in the presence of ANS, as above, and the resulting spectra after addition of 4 μg of either R_d or R_n. Native rhodanese had no effect in the quantum yield, while addition of R_d caused a large increase in fluorescence intensity 1 min after addition of the protein. Furthermore, after 15 min after addition of R_d, the initial increase in quantum yield dropped 29%. This suggests that the PS-LUV bound enzyme underwent a rapid loss of exposed hydrophobic surfaces. This loss of interactive sites may indicate why PS is less effective than CL, since these results suggest that the majority of rhodanese complexed with PS collapses rapidly into a misfolded form. Both ANS-containing PS or CL LUVs showed an ANS fluorescence

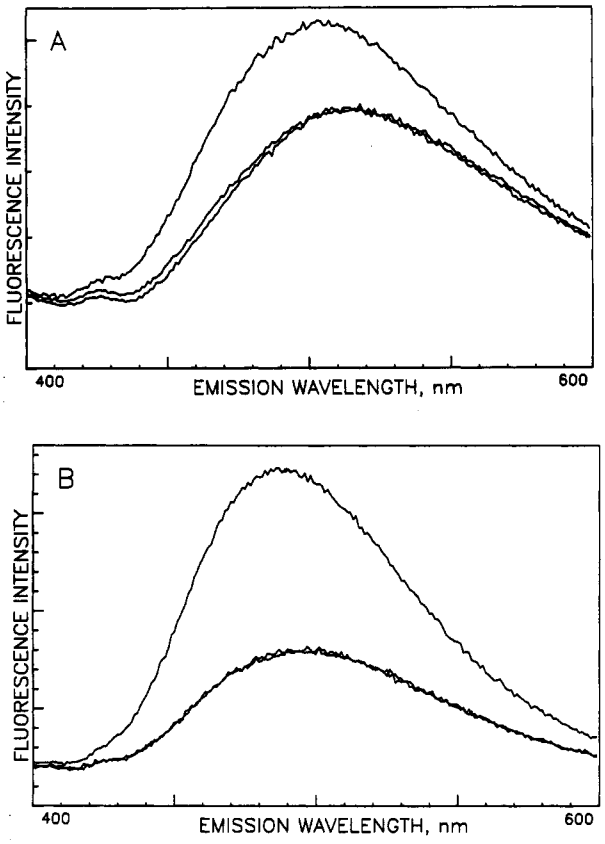

FIGURE 2: Fluorescence spectra emission of 1,8-ANS. Either native rhodanese or denatured rhodanese was added to either PS- or CL-LUVs in the presence of 20 μM ANS in buffer A, and the emission spectra from 400 to 600 nm were recorded. (Panel A) Spectra represent CL-LUV and ANS in buffer A before (lowest curve) and 1 min after addition of either R_n (middle curve), or R_d (highest curve). (Panel B) Spectra represent PS-LUV plus ANS in buffer A before (lowest curve) and 1 min after addition of either R_n (middle curve) or R_d (highest curve).

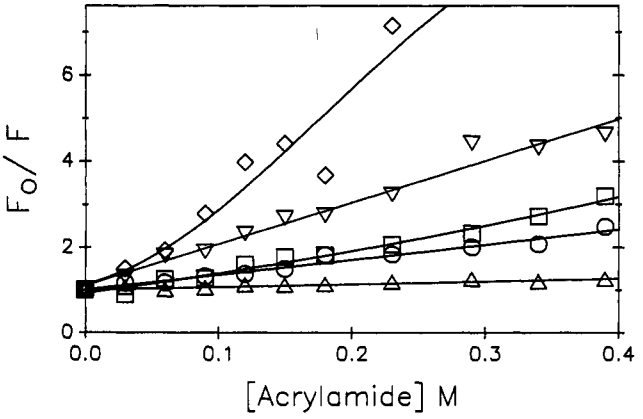

FIGURE 3: Quenching of rhodanese's intrinsic fluorescence with acrylamide. Shown is the decrease in rhodanese's intrinsic fluorescence intensity as a function of acrylamide concentration relative to a similar sample in the absence of acrylamide. The curves represent the quenching of NATA (◇), R_d (▽), R_i/PS-LUV (□), R_i/CL-LUV (○), and R_n (△).

spectra blue-shift upon addition of R_d; from 495 to 483 nm and from 515 to 483 nm, respectively.

Tryptophans in the Rhodanese–Liposome Complex Can Be Quenched to a Greater Extent Than Those in the Native Enzyme. Figures 3 and 4 show that the tryptophans in the liposome-bound rhodanese are more accessible to quenching by acrylamide and iodide, respectively, than R_n but quenched to a lesser degree than R_d. In both cases the compound NATA,

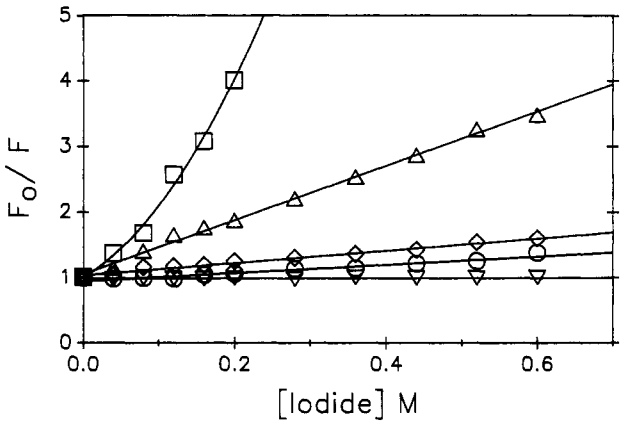

FIGURE 4: Quenching of rhodanese's intrinsic fluorescence with iodide. Shown is the decrease in the intensity of rhodanese's intrinsic fluorescence as a function of potassium iodide concentration. Curves represent the quenching of NATA (□), R_d (△), R_i/PS-LUV (◇), R_i/CL-LUV (○), and R_n (▽) carried out as described under Methods.

used for comparative purposes as a model for exposed tryptophans, was highly quenched, as expected. Figures 3 and 4 are titrations of either the rhodanese–CL-LUV complex, the rhodanese–PS-LUV complex, R_d, R_n, or NATA with either acrylamide or iodide, respectively. The data shown were plotted using the Stern–Volmer equation, and the collisional quenching constants, K_{sv}, were determined as detailed under Methods.

Quenching of R_i/LUV samples with acrylamide was carried out in a cuvette containing 3.5 μg/mL rhodanese and 90 μg/mL LUV in buffer A in a total volume of 750 μL. R_d and R_n were quenched at a similar protein concentration and reaction volume, except that no LUVs were present; and the mixtures contained 7.8 M urea in the former and 90 mM sodium phosphate buffer (pH 7.2) in the latter, instead of buffer A. Collisional quenching constants obtained when titrating with acrylamide were 9.3, 3.9, 3.1, and 0.70 M^{-1} for R_d, R_i/PS-LUV, R_i/CL-LUV, and R_n, respectively. These values indicate that both liposome-bound rhodanese forms are intermediate between the native and denatured forms of the enzyme. Furthermore, they show that there is a small, yet significant difference in the K_{sv} of R_i when bound to either PS or CL LUVs, and, as shown in Figure 3, there is also a higher degree of static quenching in the PS-LUV bound protein. The linearity of the plots at low acrylamide concentrations suggests a one-component model where all the quenching is dynamic. However, some static quenching can be detected in the liposome-bound rhodanese samples, since the plots were slightly curved at high acrylamide concentrations. The standard, NATA, used for comparative quenching purposes showed a high K_{sv} (15.9 M^{-1}) and a nonlinear curve indicating that it was susceptible to both collisional and static quenching by acrylamide.

Quenching of R_i/LUV samples with potassium iodide was carried out in a cuvette containing 4 μg/mL rhodanese, 125 μg/mL LUV, 1 mM sodium thiosulfate, and varying amounts of KI (from 0 to 0.6 M), in buffer A in a total volume of 600 μL. Additionally, the assayed mixtures were supplemented with 3 M KCl to maintain a constant ionic strength. R_n and R_d were assayed as above, except that no LUVs were present and the former contained 75 mM sodium phosphate (pH 7.2), while the latter contained 6.0 M urea, instead of buffer A. Quenching of R_d, R_i/PS-LUV, R_i/CL-LUV, and R_n with potassium iodide gave K_{sv} values of 3.7, 0.98, 0.59, and 0.20 M^{-1}, respectively. The order of quenching by iodide of the

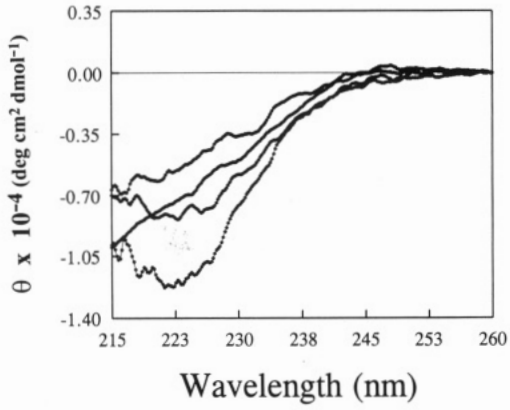

FIGURE 5: Circular dichroism spectra of rhodanese in the presence or absence of liposomes. The CD spectra represent from top to bottom at 223 nm, respectively, native rhodanese in the presence of 0.1 M sodium phosphate buffer, pH 7.5 (plot 1), or buffer A and CL-LUV (plot 2); and R_i complexed with either PS-LUV (plot 3) or CL-LUV (plot 4). Spectra was acquired as detailed under Methods and smoothed in order to identify the relative values of the mean residue molar ellipticity at 222 nm. Native rhodanese in the presence of buffer A and PS-LUV gave a spectra similar to plot 2.

different rhodanese species is the same as that seen with acrylamide. However, in this case, the intermediate species have K_{sv} values much closer to that of R_n. This was probably due to the charge of the iodide molecule which interferes with its penetration, in contrast to the uncharged acrylamide which can penetrate more easily into the inner crevices of partially folded structures, thus having greater access to inner tryptophan residues. The linearity of the plots in Figure 4, again, suggest a one-component model with only collisional quenching. The highly quenchable standard, NATA, showed a nonlinear curve with a $K_{sv} = 9.8\ M^{-1}$, indicating that it was subject to both collisional and static quenching.

Differences in the Secondary Structure of Liposome-Bound Rhodanese Compared to Native Enzyme Are Evident by Circular Dichroism Studies. Circular dichroism spectra were acquired from 530 to 215 nm for R_n, R_d, and the two R_i/LUV species. Figure 5 shows spectra obtained between 260 and 215 nm. Spectra obtained from all samples between 530 and 300 nm were similar and parallel to each other; an indication that the slight turbidity in these samples did not affect the relative results (Dorman *et al.*, 1973). The significant increase in the CD signal in the 215–235 nm range in samples which became more turbid upon addition of protein indicated that the slight amount of turbidity which developed was not enough to compromise the results since no absorption flattening distortions (Schneider, 1973) were evident. Furthermore, the A_{222} for the R_i/CL and R_i/PS complexes used in these experiments was 0.080 and 0.035, respectively, in the CD cell. Though the percent of helix in the liposome-bound rhodanese intermediates could not be empirically calculated from the molar ellipticity values due to the increase in signal produced by the presence of LUVs (compare top two curves in Figure 5), the relative amount of secondary structure in these species increased as follows: $R_n < R_n$/CL-LUV $< R_i$/PS-LUV $< R_i$/CL-LUV, based on mean residue molar ellipticity values at 222 nm. These studies show that there is a higher degree of secondary structure in the rhodanese intermediate bound to CL (lowest curve, Figure 5) than in that bound to PS liposomes (second to lowest curve, Figure 5). Native rhodanese in the presence of PS-LUV gave a CD spectra similar to that shown for R_n/CL-LUV.

Sulfhydryl Groups Are Inaccessible in Liposome-Bound Rhodanese Intermediates. Complexes of rhodanese with either CL or PS LUVs were formed, and the proteins' sulfhydryl reactivities toward the reporter probe DTNB were analyzed as detailed under Methods. DTNB was not able to react with any sulfhydryl groups in the complexed rhodanese, indicating that the SH groups were not readily accessible. However, upon treatment of the assay solution with 1% SDS, the sulfhydryl groups became accessible as indicated by a significant increase in the absorbance at 412 nm. It was difficult to accurately quantify the amount of reduced cysteines in the rhodanese's species, after addition of SDS, due to the variability of the values obtained in three independent experiments. Hence, we proceeded to determine the presence of disulfide bonded rhodanese species using SDS–PAGE.

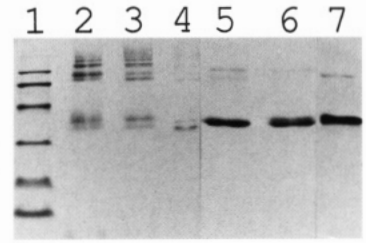

FIGURE 6: SDS–PAGE of denatured rhodanese and liposome-bound rhodanese intermediates under either nonreducing or reducing conditions. (Lane 1) Molecular weight standards (97, 66, 45, 31, 21.5, and 14.4 kDa). Lanes 2–4) R_i/CL-LUV, R_i/PS-LUV, and R_d after treatment with DTNB and electrophoresed under nonreducing conditions. (Lanes 5–7) R_i/CL-LUV, R_i/PS-LUV, and R_d under reducing conditions.

Evidence of Intra and Intermolecular Rhodanese Aggregates Are Present in R_i/Liposome Complexes. To demonstrate whether the nonreduced cysteine sulfurs in the R_i/LUVs were responsible for a lower reactivation yield due to either intra- or intermolecular disulfide formation, we treated R_d, and rhodanese, complexed with either liposome, with DTNB as above and then analyzed the proteins by SDS–PAGE under nonreducing conditions. The thionitrobenzoate group from DTNB binds covalently to reduced but not to oxidized sulfhydryls and thus prevents the oxidation of reduced sulfhydryls in subsequent analytical steps. We found that denatured rhodanese as well as both protein/liposome complexes formed a doublet in the 33-kDa range, due to intramolecular disulfide formation (Hua and Horowitz, personal communication), and each had a similar number of high molecular weight bands, which implied the presence of intermolecular disulfide-bonded species (Figure 6, lanes 2–4). Previously, we have seen that rhodanese, in the absence of liposomes, analyzed by SDS–PAGE under nonreducing conditions shows a 33-kDa doublet but only a small amount of high molecular bands (P. Horowitz, personal communication). No significant amounts of disulfide-bonded rhodanese species were detected when either R_d or R_i/liposome complexes that were not treated with DTNB were analyzed by SDS–PAGE under reducing conditions (Figure 6, lanes 5–7), supporting the above results that there exist some oxidized sulfhydryls in the liposome-bound rhodanese species which can form intra- and intermolecular disulfide bonds under nonreducing conditions. These studies show that since both liposome-bound rhodanese intermediates have a similar low percentage of reduced cysteines, disulfide bond formation is not the primary cause for the eventual differential reactivatability of either PS or CL liposome-bound rhodanese.

Turbidimetric Measurements Show That Rhodanese Intermediates Are Dynamic While Bound to Liposomes. Addition of denatured rhodanese (prepared in the absence of

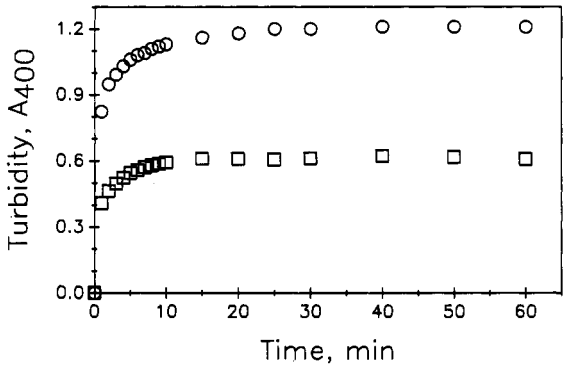

FIGURE 7: Changes in light scattering, over time, upon formation of the rhodanese liposome complex. The complex between rhodanese and either PS (○) or CL LUVs (□) was formed, and the resulting change in turbidity (light scattering) was monitored at 400 nm, over time, to show the dynamics of the complex.

β-ME) to solutions of either PS or CL LUVs in buffer A resulted in the rapid formation of a slightly cloudy suspension. The turbidity of the solution was monitored, over time, at 400 nm as detailed under Methods. The data plotted in Figure 7 show that the CL-containing sample results in greater turbidity than that with PS. This quality, however, may be an intrinsic property of the lipids, or the higher turbidity with CL LUVs may indicate that rhodanese has more binding sites for CL than for PS molecules. This would allow greater cross-linking of CL molecules by rhodanese, resulting in large aggregates which would scatter more light than a relatively smaller rhodanese–PS cluster. However, it is apparent that the turbidity of the R_i/CL-LUV complex continues to increase up to 40 min after addition of protein, while the turbidity of the R_i/PS-LUV containing solution stops increasing after 15 min. These data suggest that the rhodanese intermediate bound to CL liposomes was more dynamic than that bound to PS, and/or the high degree of light scattering suggests that the structures of both liposomes were changing. The fact that increases in light scattering was caused by either the complex and/or liposome rearrangement was further substantiated since we have observed, in control experiments, that aggregated rhodanese, at these concentrations, does not scatter light at 400 nm (data not shown).

Unfolded Rhodanese Has Multiple Binding Sites for Liposomes. To distinguish whether the increase in turbidity was due to either liposome fusion or by cross-linking of liposomes by partially folded rhodanese, we prepared the R_i/liposome complex with either PS or CL, as above, to yield a turbid solution. After the turbidity was allowed to increase to an absorbance of 0.4 at 400 nm, the samples were treated with 0.06 units of subtilisin. As proteolysis progressed, the solution became clearer. After 20 min of protease digestion, the turbidity at 400 nm was measured and found to have decreased to 0.045 and 0.056 absorbance units for CL and PS liposomes, respectively. A similar sample containing native rhodanese and liposomes had $A_{400} = 0.008$. This experiment shows that digestion of rhodanese causes a significant decrease in the samples' turbidity. This suggests that the macromolecular structure which scattered light was due to aggregation of liposomes due to cross-linking by rhodanese. Furthermore, it implies that there exist several sites in the rhodanese folding intermediate which bind to liposomes. Though there still exists the possibility that some turbidity may be due to lipid fusion, by far the majority of the turbidity seems to be caused by protein-mediated cross-linking of liposomes.

DISCUSSION

Although the characteristics of molten globules on liposomes have not previously been reported, experiments carried out in this work show that rhodanese, when complexed to either phosphatidylserine or cardiolipin liposomes, acquires structure(s) that are closely related to those defined as molten globules (Ptitsyn, 1987). Analyses carried out here for the folding intermediates are similar to the analyses for R_i bound to GroEL; though both analyses have the same interpretive difficulties, the chaperonin bound rhodanese folding intermediate is considered to be a molten globule (Martin *et al.*, 1991; Mendoza *et al.*, 1992). The molten globule is a dynamic protein folding intermediate which has regular secondary structure, is loosely-folded, yet compact, and has little or no tertiary structure, and its conformation is near native (Dolgikh *et al.*, 1981). The biophysical characteristics of rhodanese, when bound to the two LUVs used in this study, show some differences which suggest that the two folding intermediates are not identical.

Previous work in this laboratory showed that unfolded rhodanese could bind to CL-LUVs and, to a lesser extent, to PS-LUVs (Zardeneta & Horowitz, 1992c). Upon disruption of the liposome–protein complex by detergents, rhodanese's enzymatic activity could be recovered in high yields (~60%) from CL-LUV but in very low yields (<10%) from PS-LUV bound protein. This observation suggested that the more productive anchoring of rhodanese to the former LUV was due to either different conformations of the enzyme while LUV bound and/or different phospholipid binding sites in rhodanese.

Earlier, we established that the λ_{max} values for rhodanese, when bound to either CL or PS LUV were 343 and 341 nm, respectively (Zardeneta & Horowitz, 1992c). These maxima indicated that the protein species were folding intermediates, since fully denatured rhodanese has a λ_{max} at 355 nm, while native rhodanese's maxima is at 335 (Tandon & Horowitz, 1990; Martin *et al.*, 1991). Furthermore, we found that the fluorescence intensity, as well as the λ_{max}, did not change significantly over a 24 h period, when R_i was bound to either PS or CL LUVs. On the other hand, a large decrease in fluorescence quantum yield was observed for unfolded rhodanese in the presence of the nonanionic lipid phosphatidylcholine or in the absence of lipids. These results suggested that the LUV–protein complex was stabilized as an intermediate when bound to either of the two anionic lipids used in this study. In the present work, we took advantage of the stability of the complex with PS or CL LUVs to analyze the biophysical characteristics of the rhodanese species when bound to these anionic liposomes, thus shedding light on the conformations during protein folding which lead to either an inactive or active species.

Fluorescence studies with the reporter probe ANS show that there are more exposed hydrophobic surfaces in the PS-bound R_i than in the R_i complexed with CL-LUV. Additionally, quenching of both protein–lipid complexes by either acrylamide or iodide indicated that the tryptophans in rhodanese complexed with PS were more accessible to the quenchers than those in rhodanese complexed to CL-LUV. CD spectra of either complex showed that there was more secondary structure in the CL-LUV bound R_i than in the PS-LUV bound R_i, suggesting a more organized conformational state for the protein species bound to CL, relative to that bound to PS LUVs. Although there may be some shielding by liposomes, the R_i species bound to PS LUVs were slightly more resistant to subtilisin digestion than that bound

to CL LUVs; suggesting that the former species is somewhat more compact either as a near-native or as a compact misfolded conformer. Since reactivation from PS liposomes is lower than from CL-LUVs, the predominant species of R_i bound to PS liposomes is probably in a compact, misfolded conformation. In conclusion, the work reported here indicates that the R_i species bound to CL is more loosely folded than the PS-bound species, as judged by proteolysis data; however, ANS fluorescence studies and tryptophan quenching data show that PS-bound rhodanese has more hydrophobic sites and tryptophan residues exposed. These seemingly paradoxical results can be explained as follows: the conformation of each liposome-bound rhodanese species is not identical. The CL-bound species is more loosely folded in the sense that it is more easily proteolyzed yet has more secondary structure, which suggests that it is in a folding pathway which may, and does, lead to a significant amount of recovered native enzyme. In contrast, the PS-bound intermediate has less secondary structure (based on CD measurements), is more compact (based on reduced proteolytic susceptibility), yet appears to have more hydrophobic sites exposed and its tryptophans seem to be more accessible; the latter two characteristics are usually consistent with a loosely folded structure. However, the data taken as a whole suggest that the PS-bound intermediate is in a conformation that has collapsed or has misfolded and is therefore on the path toward the production of nonnative, inactive rhodanese and that the high hydrophobic exposure and tryptophan accessibility is merely a characteristic of the more compact, misfolded species bound to the PS liposomes.

It is interesting, but not surprising, that the R_i bound to either liposomes has more secondary structure than the native enzyme (either in the presence or absence of liposomes). Gierasch and others [for review, see Gierasch (1990)] have shown that lipid environments significantly increase the secondary structure of unfolded proteins. The fact that CL-LUVs increase the secondary structure of R_i more than the PS liposomes is only a reflection of the greater potential of CL-LUV to promote the organization of R_i by inducing the transition of rhodanese from a completely unfolded species to a structured, albeit loosely folded protein intermediate. In a similar manner, the different liposomes may contribute to different degrees to the observed differences in the proteins' reactivity toward ANS or tryptophan accessibility; however, again these inherent qualities of the liposomes are what promote the folding pathway which the bound protein ultimately takes.

If R_i bound to either LUV is a folding intermediate, then why does protein folding which is mediated by CL liposomes result in the recovery of active rhodanese, whereas minimal activity is recovered when it is initially sequestered by PS-LUVs? One reason may be the ability of R_i to undergo greater conformational changes while CL-LUV-bound than while PS-LUV-bound. Either liposome may initially bind a similar R_i; however, the CL-LUV bound protein undergoes a higher degree of conformational change along the path of correct folding, and, after disruption of the LUVs, the R_i which has more secondary structure and is along the correct folding pathway is the one that results in higher reactivation yields. Another reason could be that the polar head of the cardiolipin molecule, but not that of PS, binds electrostatically to certain key areas of unfolded rhodanese, keeping critical interactive surfaces apart, thus preventing misfolding. Ou *et al.* (1988) have synthesized various CL derivatives with chemical differences in their polar heads, in order to find out why several proteins that are translocated into the mitochondria bound specifically to CL-containing liposomes. They found that the presence of a free hydroxy group in the central glycerol moiety and the presence of two phosphates in the lipid head of CL were essential for the specific binding of these proteins to CL. This uniqueness of the CL molecule may, in part, explain why it may bind some protein segments in the rhodanese molecule that PS cannot. The experiments that show that the turbidity of the protein–lipid complex was decreased by protease treatment indicate that both liposomes attached to various sites on rhodanese. There are probably more binding sites in rhodanese for CL than for PS, or these sites are not identical for both lipids, thus explaining why there is a greater reactivation yield from CL liposomes.

Does this seeming specificity of CL to maintain rhodanese in a reactivatable form have any physiological significance in such events such as protein translocation across biological membranes? The ability of CL to specifically unfold and maintain another protein, cytochrome *c*, in an unfolded translocation competent conformation has been previously reported (Spooner & Watts, 1991a,b). Further, the demonstration that the amino terminus of rhodanese or that unfolded rhodanese itself can interact with and disrupt cardiolipin liposomes (Zardeneta & Horowitz, 1992b) suggests that rhodanese may possess a true signal sequence which may have a role in protein import via this interaction with the lipid. The characterization of lipid-induced conformation changes in rhodanese presented here adds to a growing body of evidence that indirectly hints at a role for CL in protein import.

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REFERENCES

Anfinsen, C. (1972) *Biochem J. 128,* 737–749.

Beckmann, R. P., Mizzen, L. A., & Welch, W. J. (1990) *Science 248,* 850–854.

Bochkareva, E. S., Lissin, L. M., Flynn, G. C., Rothman, J. E., & Girshovich, A. S. (1992) *J. Biol. Chem. 267,* 6796–6800.

Boggaram, V., Horowitz, P. M., & Waterman, M. R. (1985) *Biochem. Biophys. Res. Commun. 130,* 407–411.

Chen, W.-J., & Douglas, M. G. (1987) *J. Biol. Chem. 262,* 15605–15609.

de Jongh, H. H. J., Killian, J. A., & de Kruijff, B. (1992) *Biochemistry 31,* 1636–1643.

De Kroon, A. I. P. M., Soekarjo, M. W., De Gier, J., De Kruijff, B. (1990) *Biochemistry 29,* 8229–8240.

Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A., & Schekman, R. (1988) *Nature 332,* 800–805.

Dolgikh, D. A., Gilmanshin, R. I., Brazhnikov, E. V., Bychkova, V. E., Semisotnov, G. V., Venyaminov, S. Yu., & Ptitsyn, O. B. (1981) *FEBS Lett. 136,* 311–315.

Dorman, B. P., Hearst, J. E., & Maestre M. F. (1973) *Methods Enzymol. 27,* 767–796.

Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114,* 199–227.

Eilers, M., Endo, T., & Schatz, G. (1989) *J. Biol. Chem. 264,* 2945–2950.

Fischer, G., & Schmid, F. X. (1990) *Biochemistry 29,* 2205–2212.

Frey, S., & Tamm, L. K. (1990) *Biochem. J. 272,* 713–719.

Gething, M. J., & Sambrook, J. (1992) *Nature 355,* 33–45.

Georgopoulos, C. (1992) *Trends Biochem. Sci. 17,* 295–299.

Gierasch, L. M. (1990) in *Protein Folding: Deciphering the Second Half of the Genetic Code* (Gierasch L. M., & King, J., Eds.) pp 211–219, AAAS Press, Washington, D.C.
Horowitz, P. M. (1978) *Anal. Biochem. 86,* 751–753.
Horowitz, P. M., & Criscimagna, N. L. (1990) *J. Biol. Chem. 265,* 2576–2583.
Kim, P. S., & Baldwin, R. L. (1982) *Annu. Rev. Biochem. 51,* 459–489.
Kumamoto, C. A. (1991) *Mol. Microbiol. 5,* 19–22.
Laemmli, U. K. (1970) *Nature 227,* 680–685.
MacDonald, R. C., MacDonald, R. I., Menco, B. Ph. M., Takeshita, K., Subbarao, N. K., & Hu, L.-r. (1991) *Biochim. Biophys. Acta 1061,* 297–303.
Malikayil, J. A., Edwards, J. V., & McLean, J. R. (1992) *Biochemistry 31,* 7043–7049.
Martin, J., Langer, T., Boteva, R., Schramel, A., Horwich, A. L., & Hartl, F.-U. (1991) *Nature 352,* 36–42.
Mendoza, J. A., Lorimer, G. H., Rogers, E., & Horowitz, P. M. (1991) *J. Biol. Chem. 266,* 13044–13049.
Mendoza, J. A., Butler, M. C., & Horowitz, P. M. (1992) *J. Biol. Chem. 267,* 24648–24654.
Merril, C. R., Goldman, D., Sedman, S. A., & Ebert, M. H. (1981) *Science 211,* 1437–1438.
Miller, D. M., Delgado, R., Chirgwin, J. M., Hardies, S. C., & Horowitz. P. M. (1991) *J. Biol. Chem. 266,* 4686–4691.
Mitraki, A., & King, J. (1989) *Bio/Technology 7,* 690–696.
Nelson, R. J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M., & Craig, E. A. (1992) *Cell 71,* 97–105.
Ou, W.-J., Ito, A., Umeda, M., Inoue, K., & Omura, T. (1988) *J. Biochem. (Tokyo) 103,* 589–595.
Ploegman, J. H., Drent, G. H., Kalk., K. H., Hol, W. G. J., Heinrikson, R. L., Keim, P., Weng, L., & Russell, J. (1978) *Nature 273,* 1245–1249.
Ptitsyn, O. B. (1987) *J. Protein Chem. 6,* 273–293.
Ptitsyn, O. B., & Rashin, A. A. (1973) *Dokl. Akad. Nauk SSSR 213,* 473–475.
Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., & Schatz, G. (1986) *EMBO J. 5,* 1327–1334.
Schneider, A. S. (1973) *Methods Enzymol. 27,* 751–767.
Spooner, P. J. R., & Watts, A. (1991a) *Biochemistry 30,* 3871–3879.
Spooner, P. J. R., & Watts, A. (1991b) *Biochemistry 30,* 3880–3884.
Tandon, S., & Horowitz, P. M. (1989) *J. Biol. Chem. 264,* 9859–9866.
Tandon, S., & Horowitz, P. M. (1990) *J. Biol. Chem. 265,* 5967–5970.
Tsalkova, T., Zardeneta, G., Kudlicki, W., Kramer, G., Horowitz, P. M., & Hardesty, B. (1993) *Biochemistry 32,* 3377–3380.
Weaver, A. J., Kemple, M. D., Brauner, J. W., Mendelsohn, R., & Prendergast, F. G. (1992) *Biochemistry 31,* 1301–1313.
Williams, D. C., Van Frank, R. M., Muth, W. L., & Burnett, J. P. (1982) *Science 215,* 687–689.
Zardeneta, G., & Horowitz, P. M. (1992a) *J. Biol. Chem. 267,* 5811–5816.
Zardeneta, G., & Horowitz, P. M. (1992b) *J. Biol. Chem. 267,* 24193–24198.
Zardeneta, G., & Horowitz, P. M. (1992c) *Eur. J. Biochem. 210,* 831–837.