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Quasi-Elastic Light-Scattering Studies of Conformational States of the H,K-ATPase. Intervesicular Aggregation of Gastric Vesicles by Disulfide Cross-Linking†

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ABSTRACT: The particle size of hog gastric vesicles which contain H,K-ATPase was measured by using the method of quasi-elastic light scattering. The size of control vesicles is homogeneous as judged from its low polydispersity index. When the vesicles were treated with copper(II) *o*-phenanthroline (CuP), intervesicular S–S cross-linking occurred as determined by the aggregated vesicle size. The aggregation to divesicle size occurred very quickly, within 30 s, and the extent of aggregation did not depend on the extent of inactivation if the inactivation was not more than about 30%. Blocking of SH groups by 5,5′-dithiobis(2-nitrobenzoic acid) in the presence of Mg^{2+} prevented CuP-induced vesicular aggregation but not inactivation, indicating that S–S cross-linking rather than enzyme inactivation is the primary cause of vesicular aggregation. The presence of Mg^{2+} was required for the occurrence of aggregation. Nucleotides such as ADP ($K_{0.5}$ = 5 μM) and 5′-adenylyl imidodiphosphate ($K_{0.5}$ = 50 μM) inhibited the aggregation induced by 50 μM CuP plus 2 mM Mg^{2+} in a dose-dependent manner. Furthermore, K^+ antagonized the effects of nucleotides. The extent of aggregation increased as the pH decreased in the pH range 6.1–7.4. Virtually no cross-linking occurred at alkaline pH (e.g., pH 8–9). These data show that vesicular aggregation can be assumed to reflect the conformational state of the responsible SH group in the native enzyme.

Gastric H,K-ATPase is involved in gastric acid secretion, and the presence of the enzyme is specific to parietal cells (Ganser & Forte, 1973; Lee et al., 1974; Sachs et al., 1976). The enzyme is in gastric vesicles, and it has two different K^+ sites, an internal high-affinity site and an external low-affinity site: the external low-affinity K^+ site is competitive with ATP (Wallmark & Mardh, 1979; Wallmark et al., 1980). It has been proposed that the ATPase assumes different conformational states depending on the presence of different ligands, e.g., K^+, nucleotides, and Mg^{2+} (Schrijen et al., 1980, 1981; Van de Ven et al., 1981; Jackson et al., 1983). These pieces of information were obtained by measuring changes in inactivation by chemical modification, nucleotide binding, and fluorescence probe studies. Although conformational changes have been proposed, they have not been unequivocally established. Therefore, it is worth further study by using different, especially new methods.

In this paper, we introduce a new method to obtain information about conformational states of membrane-bound enzymes. Using copper(II) *o*-phenanthroline (CuP),[1] which

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[1] Abbreviations: CuP, copper(II) *o*-phenanthroline; AMPPNP, 5′-adenylyl imidodiphosphate; QELS, quasi-elastic light scattering; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); $NaDodSO_4$, sodium dodecyl sulfate; kDa, kilodalton(s); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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induces S–S cross-linking (Kobashi, 1968), we treated vesicles in the presence and absence of Mg^{2+}. We found that in the presence of Mg^{2+}, the CuP reaction induced intervesicular aggregation. The size of aggregated vesicles was measured by using the method of quasi-elastic light scattering. To form the intervesicular cross-links by the S–S bond, at least two sulfhydryl groups which are present on separate vesicles must project to some extent from the external surface of the ATPase or at least must exist on the outermost surface of the enzyme. Studies of $NaDodSO_4$–acrylamide gel electrophoresis of the vesicles reveal a 100-kDa protein band which comprises more than 80% of the total amount of protein and which can be phosphorylated by ATP (Saccomani et al., 1977). Therefore, the occurrence and the extent of the intervesicular S–S cross-linking must reflect the conformational states of the enzyme. The present study shows that the extent of aggregation produced by S–S cross-linking varies with the concentration of ligands for the gastric H,K-ATPase.

Materials and Methods

Gastric vesicles in 250 mM sucrose (unbuffered) were obtained from hog gastric mucosa. The details of the method were described elsewhere (Takeguchi et al., 1981). Vesicles were stored at −80 °C and used within 5 days. The activity of the H,K-ATPase was measured in a solution containing 20 μg/mL protein, 3 mM MgATP, and 40 mM Tris-HCl (pH 7.4), in the absence and presence of 15 mM KCl. The reaction was done at 37 °C for 10 min. Inorganic phosphate released was determined as described elsewhere (Yoda & Hokin, 1970). The former activity measured without K^+ is defined as Mg^{2+}-ATPase activity. The difference between activities measured with and without K^+ is defined as K^+-ATPase activity. Control K^+-ATPase activity was about 35 μmol of P_i mg^{-1} h^{-1}. All chemicals were of reagent grade.

Quasi-Elastic Light-Scattering (QELS) Method. This method has been applied to measure vesicle sizes such as sarcoplasmic reticulum (Selser et al., 1976) and islet granules (Matthews et al., 1982). The intensity of laser light scattering from the vesicle solution fluctuates due to the thermal motion of vesicle particles. We used a single clipping method to calculate the autocorrelation coefficient, $g^{(1)}(\tau)$, of the fluctuation of light scattering (Selser et al., 1976). Since the autocorrelation curve does not decay with a single exponential curve, the cumulative expansion method was used to obtain the mean decay constant (Koppel, 1972). That is

$$|g^{(1)}(\tau)| = (1 + \mu_2\tau^2/2) \exp(-\bar{\Gamma}\tau) \quad (1)$$

where τ is the lag time and μ_2 is the second expansion coefficient. Computer fitting of the experimental autocorrelation coefficients with eq 1 by using the nonlinear least-squares method gave μ_2 and $\bar{\Gamma}$, in which fitting μ_2 and $\bar{\Gamma}$ are independent variables. The value of $\mu_2/\bar{\Gamma}^2$ is the polydispersity index of the size distribution; the index equals (standard deviation/mean)^2.

The mean decay constant is related to the mean translational diffusion coefficient, $\bar{D}_{trans}$:

$$\bar{D}_{trans} = \bar{\Gamma}/K^2 \quad (2)$$

where

$$K = (4\pi n/\lambda) \sin(\theta/2) \quad (3)$$

where λ, n, and θ are the wavelength of incident light, the refractive index of solvent, and the scattered angle, respectively. The mean radius of vesicles, R, is calculated from the Stokes–Einstein equation:

$$R = kT/(6\pi\eta\bar{D}_{trans}) \quad (4)$$

where k, η, and T are the Boltzmann constant, the viscosity of solvent, and the absolute temperature, respectively.

The QELS apparatus consisted of a He–Ne laser (5 mW, λ = 6238 Å, GLG 2033, NEC), an optical cell, a photomultiplier (R649S, Hamamatzu TV, set at θ = 90°), a photon counter (C-1230, Hamamatzu TV), a pulse counter interface (Iwatsu, specially ordered), and a microcomputer (NEC PC-9801). For each experiment, 100 autocorrelation curves were obtained, and they were averaged. For control vesicles (the diameter is denoted as $\bar{D}_{un}$) and aggregated vesicles with a diameter less than about 1.8 $\bar{D}_{un}$, the sampling time of the data was about 12 s, and it took 2.5 min for calculation of the averaged autocorrelation curve. For larger aggregated vesicles whose diameters were (1.8–3.6)$\bar{D}_{un}$, the sampling time was about 22 s. The calculated curve made of 512 points decayed well within the first 48 points.

Intervesicular Cross-Linking. Stock solutions of water, electrolyte, and sucrose were filtered to remove dust particles. Control vesicle solution (0.2 mg/mL) was also filtered through a 1.0-μm polycarbonate membrane (Nuclepore Corp.). The vesicle solution included 250 mM sucrose and 4 mM Tris-HCl (pH 7.4) unless otherwise noted.

Various amounts of Mg^{2+} and CuP were added to the test solution at 25 °C to induce intervesicular cross-links. When indicated, either ADP or AMPPNP was added to the solution together with CuP in the presence and absence of K^+. The CuP concentration denotes the *o*-phenanthroline concentration, and the concentration ratio of Cu^{2+}/*o*-phenanthroline was set at 1/2 as in 25/50 and 50/100 (in micromolar). Other details are given in the figure legends.

In most experiments, we used the ratio of the particle diameter ($\bar{D}/\bar{D}_{un}$) as an index to show the extent of aggregation caused by S–S cross-linking at 25 °C, where $\bar{D}$ is the mean diameter of the particle in the presence of the cross-linking agent.

Reproducibility of the experimental value of $\bar{D}/\bar{D}_{un}$ was very good when the experiments were done on the same preparation. Every week, we prepared a new vesicle preparation for 3 years. Although some preparations gave abnormally large or small values for $\bar{D}/\bar{D}_{un}$ under the same condition, most preparations gave normal values. Therefore, all experiments were done by using at least two or three different preparations, and each figure is based on typical results of a single preparation. Experimental points in figures were fitted by hand.

$NaDodSO_4$–Polyacrylamide Slab Gel Electrophoresis. Membrane protein at 0.5 mg/mL protein was solubilized by incubating for 1 h at 25 °C in 10 mM Tris-HCl (pH 7.4) containing 1% $NaDodSO_4$, 1% β-mercaptoethanol, and 20% glycerin. Samples (15 μg of protein) were applied to each sample well of the stacking gel. The separating gel consisted of 0.1% $NaDodSO_4$, 5% acrylamide, 0.132% *N,N'*-methylenebis(acrylamide), 0.1% ammonium persulfate, 0.1% *N,N,N',N'*-tetramethylethylenediamine, and 0.375% Tris (adjusted to pH 8.8). Reservoir buffer consisted of 2.8% glycine, 1% $NaDodSO_4$, and 0.05 M Tris (pH 8.3). Electrophoresis was performed at a constant current of about 2 mA/(cm width). The gels were stained with Coomassie blue. Gel densitometry was carried out by using a dual-wavelength thin-layer chromatograph scanner (reference, 760 nm; sample, 580 nm). Molecular weights were estimated by using a high molecular weight calibration kit (Pharmacia Fine Chemicals Inc.).

Results

Size of Control Vesicles. As summarized in Table I, the average diameter of control, unaggregated vesicles in 250 mM

Table I: Average Diameter and Polydispersity Index of Control Vesicles

	average diameter[a] (μm)	polydispersity index[a]
hog gastric vesicles[b]	0.18 ± 0.03 (52)	0.026 ± 0.004 (14)[c]
hog gastric vesicles[d]	0.15 ± 0.02	0.016[e]
rabbit gastric vesicles[f]	0.60 ± 0.44	0.55[e]

[a] Mean ± SD (number of experiments). [b] Average diameter and polydispersity index are obtained from present QELS studies. [c] Defined as $\mu_2/\bar{\Gamma}^2$. [d] Average for 33 vesicles in the negative-stain electron micrograph (Figure 7; Saccomani et al., 1977). [e] Defined as (SD/mean)^2. [f] Average for 38 vesicles in the picture of freeze–fracture replicas (Figure 2; Wolosin & Forte, 1981). The vesicles were prepared from rabbit gastric mucosa which had been stimulated with histamine.

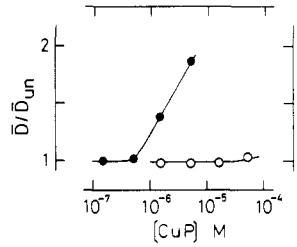

FIGURE 1: Effect of CuP concentration on the aggregation of gastric vesicles. The vesicle suspension (0.2 mg/mL protein) in 0.25 M sucrose buffered with 4 mM Tris-HCl (pH 7.4) was incubated at 25 °C for 30 min in the presence (●) and absence (○) of 5 mM Mg^{2+}. The extent of aggregation, $\bar{D}/\bar{D}_{un}$, was measured by the QELS method, where $\bar{D}_{un}$ is the mean diameter of control vesicles and $\bar{D}$ is that of aggregated vesicles.

sucrose at 25 °C was 0.18 μm ± 0.03 μm (mean ± SD) for 52 experiments. The polydispersity index, $\mu_2/\bar{\Gamma}^2$, is 0.026. From a negative-stain electron micrograph of hog gastric mucosa (Saccomani et al., 1977), the average diameter is calculated to be about 0.15 μm, and the polydispersity index, (SD/mean)^2, is about 0.02. Therefore, the diameter and the polydispersity index obtained from QELS measurements are in reasonably good agreement with those from electron microscopic studies. The small difference is likely due to the following facts. The negative-stain technique is known to cause vesicle shrinkage and shape distortion (Deamer & Baskin 1969); furthermore, QELS measurement is less sensitive to the presence of smaller particles when the preparation is not homogeneous (Briggs & Nicoli, 1980). The small polydispersity index indicates that hog gastric vesicles are tubulovesicles themselves which are seen abundantly in nonsecreting parietal cells, because vesicles which are resealed, pinched-off apical membranes usually have a larger polydispersity index. In other types of gastric vesicles which are obtained from well-stimulated rabbit gastric mucosa and supposed to be originated from the apical membrane of acid-secreting cells, their size is much larger and more heterogeneous (Wolosin & Forte, 1981), that is, the average size is calculated to be about 0.60 μm, and the polydispersity index is about 0.55 from their electron micrograph (Table I).

Mg^{2+} Is Necessary for the Occurrence of Intervesicular Aggregation. As shown in Figure 1, incubation of vesicles with CuP for 30 min at 25 °C in the absence of added Mg^{2+} [2] induced no increase in the diameter, however, in the presence of 5 mM Mg^{2+}, it induced an increase in the diameter when the CuP concentration exceeded about 1 μM. The Mg^{2+} concentration dependence of the CuP-induced aggregation is shown in Figure 2. A concentration of Mg^{2+} higher than 1 mM is required for aggregation in the presence of 10 μM CuP.

[2] Tightly bound endogenous Mg^{2+} is present (Van De Ven et al., 1981).

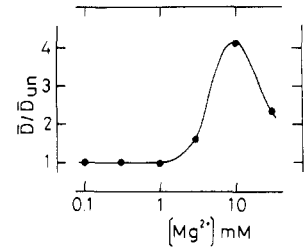

FIGURE 2: Effect of Mg^{2+} concentration on the aggregation of gastric vesicles induced by CuP. The vesicle suspension was incubated with 10 μM CuP plus various concentrations of Mg^{2+} for 30 min at 25 °C (pH 7.4).

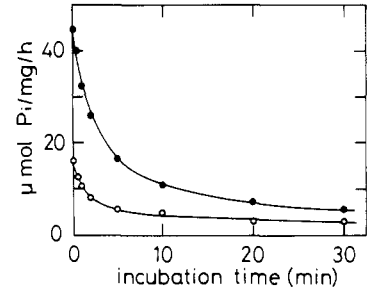

FIGURE 3: Enzyme activities of H,K-ATPase treated with CuP. Vesicles (0.2 mg/mL protein) in 0.25 M sucrose and 4 mM Tris-HCl (pH 7.4) were incubated with 20 μM CuP in the absence of Mg^{2+} for various times at 25 °C, followed by addition of 0.5 mM EDTA. The remaining enzyme activities in terms of Mg^{2+}-ATPase activity (○) and K^+-ATPase activity (●) are shown as a function of the incubation time. The activities were measured as described under Materials and Methods.

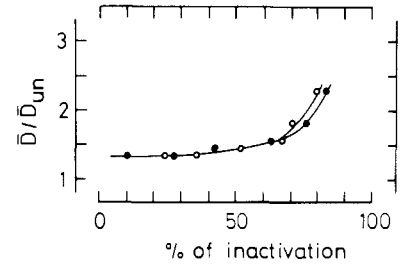

FIGURE 4: Effect of inactivation on the extent of aggregation. The inactivation in terms of Mg^{2+}-ATPase (○) and K^+-ATPase (●) activities is caused by preincubation with CuP under the conditions described in the legend for Figure 3. The vesicles preincubated with CuP were followed by another incubation with 20 μM CuP plus 5 mM Mg^{2+} for only 30 s. The aggregation reaction was terminated by addition of 0.5 mM EDTA, and then the particle size was measured.

The presence of Mg^{2+} has been considered to cause change in the conformation of the enzyme (Schrijen et al., 1980, 1981; Van De Ven et al., 1981). Present results suggest that such conformational change projects some specific SH groups into more appropriate positions for making intervesicular cross-linkings.

CuP-Induced Inactivation and Aggregation. It is necessary to know whether conformation is related to native ATPase or inactivated ATPase, because incubation of vesicles with CuP also induces inactivation of H,K-ATPase. For this purpose, the extent of inactivation and the extent of formation of intervesicular cross-links were compared. In the experiments shown in Figure 3, the vesicles were preincubated with 20 μM CuP in the absence of Mg^{2+} for various times at 25 °C, and then the reaction was terminated by addition of 500 μM EDTA. The remaining activities in terms of Mg^{2+}- and K^+-ATPase activities were assayed. In parallel experiments, the vesicles were preincubated with 20 μM CuP in the absence of Mg^{2+} for various times and then preincubated for 30 s with

20 μM CuP plus 5 mM Mg^{2+}, followed by immediate addition of 0.5 mM EDTA. It is interesting that a reaction as short as 30 s induces significant aggregation. Figure 4 shows the ratio of $\bar{D}/\bar{D}_{un}$ as a function of the extent of inactivation caused by preincubation with CuP. Both Mg^{2+}-ATPase and K^+-ATPase activities give a similar relationship. There was no parallel relationship between the extent of inactivation and $\bar{D}/\bar{D}_{un}$ when the inactivation was less than about 30%, and the value of $\bar{D}/\bar{D}_{un}$ was almost constant. The extrapolated value for $\bar{D}/\bar{D}_{un}$ to zero inactivation was about 1.3. As discussed later, the value of 1.3 means significant aggregation. On the other hand, highly inactivated states of more 70% inactivation accompanied higher $\bar{D}/\bar{D}_{un}$ values (Figure 4).

These results suggest that vesicular aggregation occurs in two phases: in the first phase, small but significant aggregation occurs with little enzyme inactivation; in the second phase, extensive aggregation occurs concurrently with considerable enzyme inactivation. It should be noted, however, that vesicular aggregation in this second phase is not the consequence of enzyme inactivation per se, since aggregation but not inactivation was prevented by pretreatment of vesicles with butanedione (see below).

Furthermore, the constancy of $\bar{D}/\bar{D}_{un}$ which was observed when inactivation was less than about 30% suggests that the conformational state of the partially inhibited enzyme is the same as that of the native enzyme as discussed later.

The intervesicular cross-linkings, even at the extent of 1.3 for $\bar{D}/\bar{D}_{un}$, could not be reversed by incubation with 1 mM dithiothreitol for 1 h at 25 °C, as judged from QELS measurements.

Effects of Butanedione on Aggregation. The relationship between the conformational state of the enzyme and the inactivation described above is not general but specific to the CuP reaction. For example, experiments using butanedione, which modifies arginine residues of H,K-ATPase (Schrijen et al., 1979), revealed a different relationship. Pretreatment of the enzyme (0.2 mg/mL protein) with 1 mM butanedione for 1 h at 25 °C in a solution containing 40 mM sodium borate, 250 mM sucrose, and 2 mM Mg^{2+} led to inactivation of enzyme activity (22% of the control activity remained). Cross-linking reaction by 50 μM CuP plus 2 mM Mg^{2+} of the butanedione-treated vesicles for up to 30 min in the same buffer failed to induce aggregation of vesicles, whereas control vesicles in the same borate buffer can be aggregated by CuP. Therefore, the conformational state of the butanedione-treated vesicles is different from that of the CuP-treated enzyme.

$NaDodSO_4$–Acrylamide Slab Gel Electrophoresis of Aggregated Vesicles. Gel electrophoresis patterns of control and CuP-treated vesicles are compared. As reported recently (Takeguchi et al., 1983), treatment of vesicles with CuP in the absence of Mg^{2+} opens the KCl pathway across the gastric vesicle membrane. It seems to afford a biochemical basis for the morphological change of parietal cells from the resting to the secreting state. Treatment of vesicles with 50 μM CuP at 25 °C for 30 min in the absence of Mg^{2+} was followed by addition of 1 mM EDTA. It led to the formation of a new 220-kDa band and a decrease of the 105-kDa band (Figure 5A). The new band can be a candidate for the increase in KCl permeability and for inactivation of enzyme activity. Next, the S–S cross-linking reaction was done in the presence of 5 mM Mg^{2+} under the same conditions. The value of $\bar{D}/\bar{D}_{un}$ was large, about 4. In Figure 5B, we can see clear differences between those vesicles treated with CuP in the presence and absence of Mg^{2+}. In addition of 105- and 220-kDa bands, a new 310-kDa band and more highly cross-linked bands are

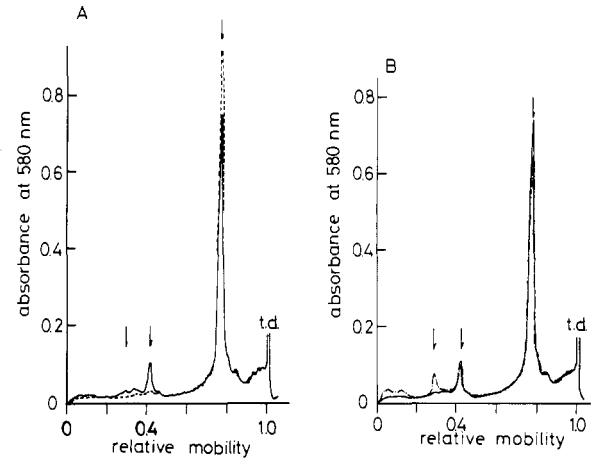

FIGURE 5: Effect of CuP on the pattern of $NaDodSO_4$–acrylamide gel electrophoresis of gastric vesicles. Solubilization of the enzyme and gel electrophoresis were as described under Materials and Methods. (A) Control vesicles (---); vesicles treated with 50 μM CuP for 30 min in the absence of Mg^{2+} (—). (B) Vesicles treated with 50 μM CuP for 30 min in the absence of Mg^{2+} (—) and in the presence of 5 mM Mg^{2+} (-·-). t.d. indicates the position of the tracking dye. (—) designates the 105-, 220-, and 310-kDa polypeptides.

seen on the gel of vesicles treated in the presence of Mg^{2+}. The difference shows that interpolypeptide cross-linking engages in the aggregation.

When 5 μM CuP was used in exchange for 50 μM CuP ($\bar{D}/\bar{D}_{un}$ was 1.3 in the presence of 5 mM Mg^{2+}), the 310-kDa band and more highly cross-linked bands diminished completely; furthermore, the area of the 220-kDa band on the gel obtained from the aggregated vesicles was about 30% larger than that obtained from the CuP-treated unaggregated vesicles. Present findings indicate that a small fraction of the total polypeptides on each gastric vesicle engages in the intervesicular cross-linking and that 220- and 310-kDa polypeptides and more highly cross-linked polypeptides engage in aggregation.

Effects of Nbs_2 Pretreatment of Vesicles on Formation of Intervesicular Aggregation: Characterization of the SH Groups. When the enzyme is modified with Nbs_2 in the absence of Mg^{2+}, it is known that vital sulfhydryl groups which are involved in K^+-sensitive dephosphorylation are modified, whereas in the presence of Mg^{2+}, the K^+-dependent phosphorylation is not affected by Nbs_2 reaction but sulfhydryl groups which are involved in phosphorylation are modified (Schrijen et al., 1981).

We did experiments to determine which type of sulfhydryl group engages in intervesicular cross-linkings. Vesicles (1.0 mg/mL) were preincubated with Nbs_2 for 30 min at 37 °C in a solution containing 250 mM sucrose and 20 mM Tris-HCl (pH 7.4) in the presence or absence of 2 mM Mg^{2+}. Separations of the enzyme preparations from these Nbs_2 reaction mixtures were performed by gel filtration at 4 °C over a column of Sephadex G-25. For elution, buffers containing 250 mM sucrose and 4 mM Tris-HCl (pH 7.4) with or without 2 mM Mg^{2+} were used, respectively. Then 2 mM Mg^{2+} was added to the latter vesicle eluate. The two preparations were incubated with 50 μM CuP for 5 and 10 min at 25 °C to form intervesicular S–S cross-links. The value of $\bar{D}/\bar{D}_{un}$ for the preparation which had been modified with Nbs_2 in the absence of Mg^{2+} showed the occurrence of aggregation; that is, the value increased to 1.3 and 1.5 for the 5- and 10-min CuP reactions, respectively. On the other hand, preincubation of vesicles with Nbs_2 in the presence of Mg^{2+} prevented the formation of intervesicular cross-links by CuP. These results

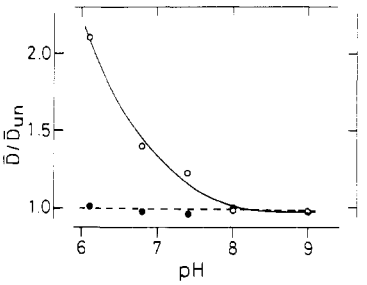

FIGURE 6: Effect of pH on the aggregation of gastric vesicles caused by CuP. The vesicle suspension (0.2 mg/mL protein) was incubated with 25 μM CuP at various pHs in 0.25 M sucrose in the presence (○) and absence (●) of 2 mM Mg^{2+}. The medium buffer was 4 mM Tris–Pipes at pH 6.1, 6.8, and 7.4, and 4 mM Tris-HCl at pH 8.0 and 9.0.

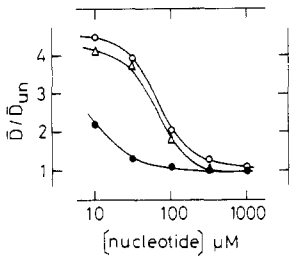

FIGURE 7: Effect of [nucleotide] and $[K^+]$ on the aggregation of vesicles caused by CuP. The vesicle suspension was incubated with 50 μM CuP plus 2 mM Mg^{2+} containing various concentrations of ADP (●) and AMPPNP (△) for 30 min at 25 °C (pH 7.4). For the experiments (○), the concentration of ADP was changed in the presence of 50 mM K^+.

suggest that sulfhydryl groups which are involved in phosphorylation engage in CuP-induced aggregation.

Effects of pH on Aggregation by CuP. The extent of aggregation caused by incubation with 25 μM CuP plus 2 mM Mg^{2+} for 30 min at 25 °C is found to be a function of the medium pH (Figure 6). From pH 6.1 to 9.0, $\bar{D}_{un}$ was constant and independent of the presence or absence of 2 mM Mg^{2+}. At alkaline pH (≥8), aggregation caused by 25 μM CuP was completely diminished. Since a model compound of cysteine is oxidized increasingly as the medium pH increases (Kobashi, 1968), the increased ability to form intervesicular cross-links at acidic pHs is considered to reflect the conformational state of the enzyme.

Prevention of CuP-Induced Vesicle Aggregation by Nucleotide and Antagonistic Effects of K^+. Figure 7 shows effects of nondegradable nucleotides such as ADP and AMPPNP on the formation of intervesicular S–S cross-links by 50 μM CuP plus 2 mM Mg^{2+}. (Experiments using ATP were not done because ATP at low concentrations was quickly digested under these conditions.) Since nucleotides bind to the ATP site of the enzyme with a dissociation constant of 50 μM for the AMPPNP–enzyme complex (Van De Ven et al., 1981), the prevention of aggregation suggests that nucleotide binding antagonizes the Mg^{2+}-induced conformational change. The half-inhibition took place at 50 μM for AMPPNP and at 5 μM for ADP.

The effects of nucleotides greatly decrease in the presence of K^+ (Figure 7). From the concentration dependence of K^+ on the antagonistic effect (data not shown), the value of $K_{0.5}$ was 32 mM in the presence of 60 μM ADP. Furthermore, the extent of aggregation caused by 50 μM CuP plus 2 mM Mg^{2+} in the absence of nucleotides did not depend on the K^+ concentration in the range from 1 to 100 mM under the condition of constant osmolarity. This indicates that K^+ reduces the amount of the nucleotide bound.

Discussion

We compared the average size and the polydispersity index of control vesicles determined by the QELS method with corresponding values determined by the electron microscopic method. Results from both methods are in good agreement. The polydispersity index of present vesicles is about one-twentieth that of stimulation-associated gastric vesicles which are resealed, pinched-off apical membrane vesicles (Wolosin & Forte, 1981). The low polydispersity index is further evidence which supports that hog gastric vesicles are naturally occurring tubulovesicles seen abundantly in the resting parietal cell, as recently suggested from antibody staining (Smolka et al., 1983). By contrast, pinched-off cytoplasmic vesicles (e.g., sarcoplasmic reticulum) have a large polydispersity index, ranging from 0.2 to 0.7 (Selser et al., 1976).

When gastric vesicles are treated with the S–S cross-linking reagent CuP in the presence of Mg^{2+}, vesicle aggregation takes place. Studies of $NaDodSO_4$–acrylamide gel electrophoresis show that interpolypeptide cross-linkings engage in the aggregation. The effect of Mg^{2+} suggests that Mg^{2+}-induced conformational change in the enzyme is prerequisite to intervesicular cross-linking. Neutralization of the surface negative charge of the vesicles may be induced by the addition of Mg^{2+}. However, the following facts suggest that neutralization is at least not a determining factor for inducing aggregation. After modification of arginine groups with butanedione, which inhibits enzyme activity, the addition of CuP plus Mg^{2+} induced no aggregation. Furthermore, the presence of Mg^{2+} higher than about 10 mM decreases the extent of aggregation (Figure 2).

An interesting, important relationship was obtained between the extent of aggregation and the extent of inactivation (Figure 4). The 30-s aggregation reaction increased the value of $\bar{D}/\bar{D}_{un}$ to 1.3, irrespective of the extent of inactivation if the percent inactivation was less than about 30%. Since there is no correlation between them, the occurrence of aggregation reflects the conformational state of the native enzyme.

When two vesicles are cross-linked and have thermal motion as one particle in a solution, the diameter of the equivalent spherical aggregate is calculated to be about 1.3 times the diameter of the unaggregated vesicle by use of Einstein's hydrodynamic theory. Therefore, the rapid increase in the diameter caused by the short aggregation reaction indicates the occurrence of significant aggregation between two vesicles. Irreversibility of intervesicular cross-linkings by dithiothreitol shows that the close contact of the two vesicles protects the cross-links against attack by dithiothreitol, possibly due to steric hindrance.

To form an intervesicular S–S cross-link by CuP reaction, two SH groups on different vesicles are required to come within several angstroms of each other. As the extent of inactivation becomes larger, the possibility decreases that the two SH groups belong to native enzymes or it increases that both belong to inactivated enzymes, as estimated from the theory of random collision probability. As the extent of inactivation becomes larger, $\bar{D}/\bar{D}_{un}$ caused by the 30-s CuP reaction would decrease if cross-linking had occurred only between native enzymes or $\bar{D}/\bar{D}_{un}$ would increase if cross-linking occurred only between inactivated enzymes. However, these are not the case when inactivation was no more than about 30% (Figure 4). The constancy of $\bar{D}/\bar{D}_{un}$ indicates that no discrimination between native and inactivated enzymes occurred in the formation of S–S cross-links. One reasonable explanation for the fact is that the conformational state of the partially inactivated enzyme is the same as that of the native enzyme. Since several

SH groups per molecule exist and formation of multiple intramolecular S–S cross-links may be possible (Saccomani et al., 1975; Schrijen et al., 1979; Forte et al., 1981; Nandi et al., 1983), the initial partial inactivation is considered to be due to the S–S reaction which does not change the conformational state of the enzyme.

When the inactivation was greater than about 70%, $\bar{D}/\bar{D}_{un}$ progressively increased as the percent inactivation increased (Figure 4). The inactivation of the enzyme does not deprive it of the ability to aggregate; on the contrary, it enhances the ability. This means that the SH groups responsible for aggregation are protected against the S–S cross-linking agent in the absence of Mg^{2+} and addition of Mg^{2+} can induce a conformational change in the inhibited enzyme in the same direction as that found in the native enzyme. This is quite different from the case of butanedione, which reacts with arginine residues.

Besides the requirement of Mg^{2+} for the S–S cross-linking-mediated vesicular aggregation described above, several elements have crucial effects on intervesicular cross-linking. For instance, some nucleotides such as ADP and AMPPNP antagonize the effect of Mg^{2+}. Furthermore, the inhibitory effects of nucleotides are reversed by K^+ ($K_{0.5}$ = 32 mM for ADP). The formation of cross-links depends on the medium pH. The pH dependence is quite similar to that of the reactivity of a specific inhibitor of H,K-ATPase, picoprazole (Wallmark et al., 1983). These facts suggest that formation of the intervesicular S–S cross-links depends on the conformational states of the specific SH groups. Thus, the quasi-elastic light scattering method described here is a useful tool for the studies of conformational states of H,K-ATPase.

Registry No. ATPase, 9000-83-3; ADP, 58-64-0; AMPPNP, 25612-73-1; K, 7440-09-7; Mg, 7439-95-4.

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