Determination of the Distribution of Sodium and Potassium Ion Activated Adenosinetriphosphatase among the Various Oligomers Formed in Solutions of Nonionic Detergents†

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ABSTRACT: Sodium and potassium ion activated adenosinetriphosphatase [($Na^+ + K^+$)-ATPase] can be dispersed from the membrane-bound state, with the stable retention of the capacity to display ($Na^+ + K^+$)-ATPase activity, by treatment with solutions of a homogeneous, nonionic detergent, octaethylene glycol dodecyl ether. The dispersed enzyme is incapable of turnover, however, in solutions where the free detergent concentration is above the critical micelle concentration. Treatment of solutions of this enzyme with the cross-linking reagent glutaraldehyde results in the quantitative, covalent coupling of the α- and β-polypeptides. The various covalent products formed, when visualized on sodium dodecyl sulfate–polyacrylamide gels, are integral oligomers of the asymmetric unit ($\alpha\beta$) of the enzyme. The noncovalent oligomers from which these products are derived can be separated on sucrose gradients based on differences in their respective sedimentation coefficients, but these sedimentation coefficients are highly dependent on the concentration of detergent in the gradient. Furthermore, the cross-linking assay reveals that changes in the aggregation state of the enzyme occur as detergent:protein ratios are varied or when the enzyme is added to the ATPase assay. These observations suggest that earlier conclusions about the oligomers of this enzyme present in detergent solution were significantly in error.

The two polypeptide chains, designated α and β, that constitute sodium and potassium ion activated adenosinetriphosphatase [($Na^+ + K^+$)-ATPase][1] have been shown to exist in an equimolar ratio in purified preparations of the enzyme and to be part of the same molecular complex because they can be cross-linked quantitatively to form a unique covalent heterodimer, α-β (Kyte, 1972; Liang & Winter, 1977; Craig & Kyte, 1980). An earlier report from this laboratory (Craig & Kyte, 1980) estimated the protein molecular weight of this $\alpha\beta$ unit to be 177 000 ± 13 000, but this is a somewhat controversial value (Peterson & Hokin, 1981). Other cross-linking results have suggested that an α_2 complex may be present in the native structure of the enzyme (Kyte, 1975; Giotta, 1976; Liang & Winter, 1977). In these cases, however, quantitative cross-linking was never achieved, nor was the problem of random collisional cross-linking within the membrane (Kyte, 1981) addressed satisfactorily, leaving in doubt the question of the oligomeric state of the enzyme within the membrane.

There are reports that the treatment of membrane-bound ($Na^+ + K^+$)-ATPase with certain nonionic detergents results in the formation of apparently discrete, soluble complexes that have the capacity to display ATPase activity. The protein molecular weights of these complexes have been estimated to be 380 000 ± 21 000 in solutions of Lubrol WX (Hastings & Reynolds, 1979) and either 256 000 ± 23 000 (Esmann et al., 1980) or 170 000 ± 9000 (Brotherus et al., 1981) in solutions of octaethylene glycol dodecyl ether ($C_{12}E_8$). In addition, the protein portions of complexes formed in solutions of Triton X-100 or digitonin have been reported to have molecular weights of 140 000 (Clarke, 1975) and 190 000 ± 24 000 (Winter & Moss, 1979), respectively, although the enzyme is inactivated by each of these detergents. All of the molecular weights determined in the presence of these detergents depend on evaluations in which very substantial corrections are necessary in order to derive the final molecular weights from the hydrodynamic properties, a fact that may explain why values that must be simple, integral multiples of a unique number lying between 140 000 and 180 000 display such discrepancy.

This report describes several properties of ($Na^+ + K^+$)-ATPase when it is dissolved in solutions of the nonionic, homogeneous detergent $C_{12}E_8$. For clarification of disagreements about the composition of solutions of this enzyme and this detergent (Esmann et al., 1980; Brotherus et al., 1981), a cross-linking assay has been used that can assess accurately the proportion of each quaternary complex present in any given sample (Hermann et al., 1981) and can detect with high sensitivity the changes brought about by variations of the solution.

Experimental Procedures

Materials. Octaethylene glycol dodecyl ether ($C_{12}E_8$) was obtained commercially from Nikkol Chemical Co. or was purified from Brij 36T by a combination of high-pressure liquid chromatography and conventional column chromatography on silica gel. The Brij detergents 36T, 58, 56, and 78 and Lubrol WX were purchased from Sigma Chemical Co. Glutaraldehyde was obtained from Polysciences and was a gift of Dr. K. Tokuyasu, University of California, San Diego. Sedimentation velocity standards used for sucrose gradients, listed with their sources and sedimentation coefficients, were as follows: bovine serum albumin (Pentex), 4.6 S; human transferrin (gift of Paul Saltman, University of California, San Diego), 6.1 S; rabbit muscle lactate dehydrogenase (Sigma), 7.2 S; bovine catalase (Sigma), 11.2 S; and aspartate transcarbamylase (gift of H. K. Schachman, University of Cali-

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[1] Abbreviations: ($Na^+ + K^+$)-ATPase, sodium and potassium ion activated adenosinetriphosphatase; $C_{12}E_8$, octaethylene glycol dodecyl ether (3,6,9,12,15,18,21,24-octaoxahexatriacontan-1-ol); $NaDodSO_4$, sodium dodecyl sulfate.

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fornia, Berkeley), 11.7 S. Bovine serum albumin, ovalbumin (Sigma Chemical Co.), and phosphorylase *a* (gift of James Stull, University of California, San Diego) were used as polypeptide standards for gel electrophoresis.

Enzyme and Enzymatic Assay. Purified ($Na^+ + K^+$)-ATPase was prepared by published procedures from canine renal medulla by incubating a microsomal preparation with sodium dodecyl sulfate ($NaDodSO_4$) and isolating membrane fragments of a particular density on a discontinuous sucrose gradient (Jørgensen, 1974; Munson, 1981). The usual specific activity of the purified, membrane-bound enzyme was 900–1100 μmol of P_i (mg of protein)$^{-1}$ h^{-1}, and $NaDodSO_4$ gels of the preparation contained only the α and β subunits of ($Na^+ + K^+$)-ATPase.

The method of assay for strophanthidin-sensitive ($Na^+ + K^+$)-ATPase activity has been described (Kyte, 1971). It was modified to eliminate interference from nonionic detergents during the formation of the phosphate–molybdate complex by the method of Peterson (1978). Enzyme dispersed in detergent was assayed by direct addition either into detergent-free medium such that the diluted detergent concentration was below its critical micelle concentration or into assay medium containing added detergent. Product formation was consistently linear over the reaction period of the assay. Protein determination was by the Lowry method whose extinction coefficients were determined by quantitative amino acid analysis as previously described (Kyte, 1971).

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gels were prepared with 3.6% acrylamide and 0.1% methylenebis(acrylamide) according to Weber & Osborn (1969). Samples were prepared by the addition of an amount of 20% $NaDodSO_4$ sufficient to provide at least a 5-fold weight excess over protein, incubated 12 h at 22 °C, and submitted to electrophoresis. Gels were stained and destained and then scanned, and peak areas were integrated as previously described (Craig & Kyte, 1980). Noncovalent bovine serum albumin polymers were formed by denaturing 2–3 μL of a 100 mg ml^{-1} solution of the protein with an equal volume of 20% $NaDodSO_4$. The volume was then raised to 50 μL by the addition of 0.1% $NaDodSO_4$, and the sample was submitted to electrophoresis.

Preparation of Supernatant–$C_{12}E_8$ ($Na^+ + K^+$)-ATPase. Purified, membrane-bound ($Na^+ + K^+$)-ATPase (4 mg mL^{-1} in 20 mM histidinium chloride or 30 mM imidazolium chloride, pH 7.0) is diluted at room temperature (22 °C) into 20 mM histidinium chloride or 30 mM imidazolium chloride, pH 7.0, such that the final concentrations are 1 mg mL^{-1} protein, 0.1 M KCl, and 10% glycerol, and the total, initial $C_{12}E_8$ concentration is 5.6 mM (mg of protein mL^{-1})$^{-1}$ unless otherwise stated. This corresponds to a detergent:protein weight ratio of 3 mg of detergent (mg of protein)$^{-1}$. Samples are then submitted to centrifugation at 100000*g* for 90 min at room temperature in a Beckman Airfuge to remove undissolved membranes. The pelleted material tends to be flocculent, and for this reason, samples larger than 0.5 mL are placed on a 30% sucrose cushion and submitted to centrifugation at 150000*g* for 90 min at 12 °C in a type Ti-60 Beckman rotor. The clear supernate is removed from above the cloudy interface and then assayed for ($Na^+ + K^+$)-ATPase activity [1000–1300 μmol of P_i (mg of protein)$^{-1}$ h^{-1}] and protein concentration (0.5–0.7 mg mL^{-1}). Since much of the detergent is lost with the pellet, the notation <5.6 mM $C_{12}E_8$ has been adopted when referring to the detergent concentration in the supernate.

Cross-Linking of Supernatant–$C_{12}E_8$ Enzyme. Supernatant–$C_{12}E_8$ enzyme is prepared in 30 mM imidazolium chloride. Cross-linking is initiated by the addition of 0.1 volume of 80 mM glutaraldehyde, the incubation proceeds for 45 min at room temperature, and the reaction is quenched with 0.1 volume of 0.6 M glycine, pH 9.0. After 5 min, 20% $NaDodSO_4$ [>5 mg of $NaDodSO_4$ (mg of protein)$^{-1}$] is added to denature the polypeptides, and the sample is incubated at room temperature for 12 h before being submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis.

In some experiments, the protein concentration was varied over the range 0.05–0.5 mg mL^{-1} by diluting supernatant–$C_{12}E_8$ enzyme (0.5 mg mL^{-1} protein and <5.6 mM $C_{12}E_8$) with 1 mM $C_{12}E_8$, incubating 15 min at room temperature, and cross-linking with 8 mM glutaraldehyde. In separate experiments, glutaraldehyde concentration was varied between 2 and 16 mM, incubation time between 5 and 90 min, and temperature of incubation between 10 and 30 °C. The reaction was quenched, and samples were denatured and subjected to electrophoresis.

Cross-linking was also performed under conditions where the enzyme had been determined to be turning over by prior dilution of a 50-μL sample of supernatant–$C_{12}E_8$ enzyme (0.2 mg of protein mL^{-1}) into 0.3 mL of 30 mM imidazolium chloride, pH 7.1, prepared such that the final concentrations would be 3 mM MgATP, 100 mM NaCl, and 20 mM KCl. After a 5-min incubation at 22 °C, the samples to be analyzed were cross-linked and submitted to electrophoresis. Duplicate samples, lacking glutaraldehyde, were assayed for enzymatic activity, simultaneously.

Separation of Oligomers on Sucrose Gradients Containing Detergent. Samples (100 μL) of supernatant–$C_{12}E_8$ ($Na^+ + K^+$)-ATPase at protein concentrations of either 5.8, 2.6, or 1.6 mg mL^{-1}, all prepared with 7.4 μmol of $C_{12}E_8$ (mg of protein)$^{-1}$, were loaded onto 5–20% sucrose gradients (3.6 mL) containing 30 mM imidazolium chloride, pH 7.0, and 2, 9, or 16 mM $C_{12}E_8$, respectively. The gradients (path length 5.5 cm) were submitted to centrifugation at 55000 rpm in an SW 60 rotor at 15 °C for 5.5, 6, or 6.5 h, respectively. After centrifugation, the bottoms of the tubes were punctured, fractions (approximately 100 μL) were collected, and each fraction was reacted with 8 mM glutaraldehyde for 60 min at room temperature. After the reaction was quenched with glycine, the polypeptides were denatured for electrophoresis with $NaDodSO_4$. The stained gels were scanned at 550 nm, and the relative amount of each polymer in each fraction was determined by calculating the area of its cross-linked product on each gel. The positions of the sedimentation velocity standards in companion gradients were determined by measuring A_{280} of individual fractions. In the case of lactate dehydrogenase and catalase, the positions were verified by assaying for the appropriate enzymatic activity either by the decrease in A_{340} of a solution of 2 mM sodium pyruvate and 150 μM NADH or by hydrogen peroxide consumption determined by titration with permanganate, respectively.

Results

Activity of ($Na^+ + K^+$)-ATPase Dispersed in $C_{12}E_8$. Purified, membrane-bound ($Na^+ + K^+$)-ATPase, at constant protein concentration, was mixed with increasing amounts of $C_{12}E_8$, and undissolved membranes were removed by centrifugation. Aliquots of the supernates were diluted into detergent-free assay medium to determine strophanthidin-sensitive ($Na^+ + K^+$)-ATPase activity under conditions where the amount of detergent carried over into the assay remained below the critical micelle concentration. From the results plotted in Figure 1, it can be seen that the enzyme retains the ability to display activity after it has been dispersed at high

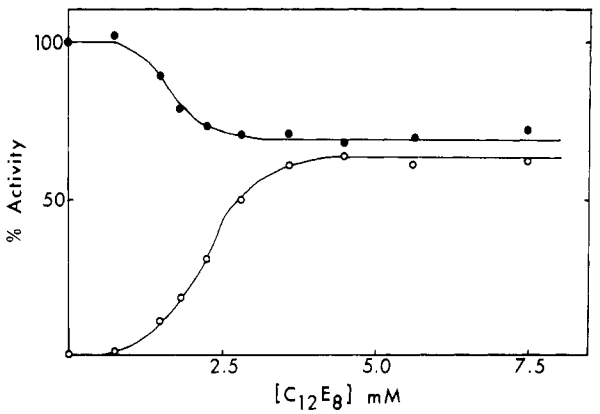

FIGURE 1: Total and supernatant activity as a function of $C_{12}E_8$ concentration. Purified membrane-bound $(Na^+ + K^+)$-ATPase was added to a solution that had been prepared so that the final concentrations would be 20 mM histidinium chloride, pH 7.0, 0.1 M KCl, 10% glycerol, 1.0 mg of protein mL^{-1}, and $C_{12}E_8$ concentration as indicated. After a 10-min incubation, samples were submitted to centrifugation at 22 °C and 100000*g* for 90 min. The supernates were removed and assayed for $(Na^+ + K^+)$-ATPase activity in a detergent-free medium. Samples removed prior to centrifugation were assayed in an identical manner and represent total activity (●) relative to that for a mixture with no $C_{12}E_8$. The activities of the supernates (○) are plotted as percent of the activity in the total mixture.

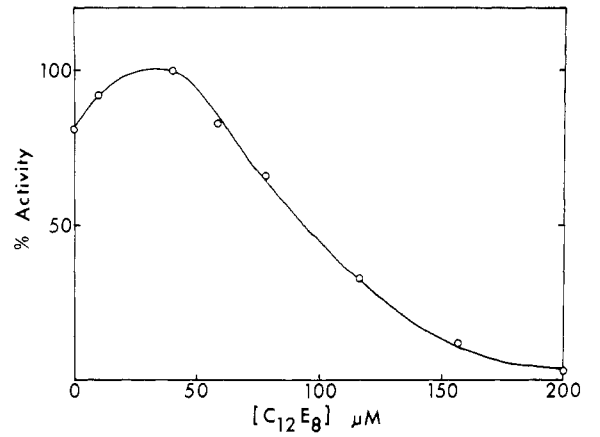

FIGURE 2: $(Na^+ + K^+)$-ATPase activity in the presence of increasing concentrations of $C_{12}E_8$. Supernatant–$C_{12}E_8$ enzyme (5 μL of 0.5 mg of protein mL^{-1} and <5.6 mM $C_{12}E_8$) was added to 0.5 mL of assay medium containing 30 mM histidinium chloride, pH 6.8, 3 mM MgATP, 100 mM NaCl, 20 mM KCl, and the indicated concentrations of additional $C_{12}E_8$. The mixtures were incubated for 6 min at 37 °C, and the ATPase reaction was quenched with $NaDodSO_4$. The activities are plotted as percent of the maximum specific activity of 920 μmol of P_i mg^{-1} h^{-1} found in the mixture containing 40 μM added $C_{12}E_8$.

detergent concentrations even though it no longer sediments at 100000*g* over 90 min. In addition, the enzyme showed no detectable (<8%) loss of activity when assayed over 24, 48, or 100 h of storage at 22 °C. Therefore, in $C_{12}E_8$, the enzyme exists as a stable supernatant complex or complexes capable of displaying activity upon dilution.

This supernatant–$C_{12}E_8$ enzyme was diluted into assay media containing several concentrations of $C_{12}E_8$ in order to test the effect of free detergent concentration on activity (Figure 2). Although $(Na^+ + K^+)$-ATPase activity is slightly increased by low levels of detergent, as the free detergent concentration is increased above the critical micelle concentration of 90 μM (Helenius et al., 1979), the enzyme activity decreases to zero. This result suggests that, although activity can be obtained when the detergent is sufficiently diluted, the enzyme is nonetheless incapable of turnover in the presence of micellar $C_{12}E_8$.

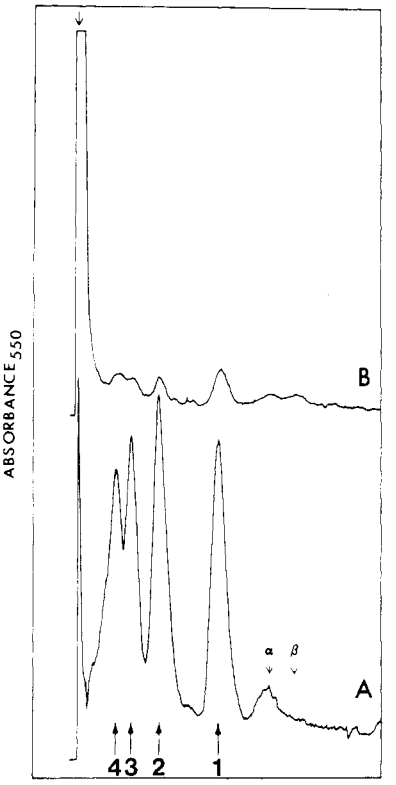

FIGURE 3: Scans of $NaDodSO_4$–polyacrylamide gels of $(Na^+ + K^+)$-ATPase cross-linked with glutaraldehyde in $C_{12}E_8$ solution (A) and in the membrane-bound form (B). A sample of supernatant–$C_{12}E_8$ enzyme (50 μL of 0.6 mg of protein mL^{-1} and <5.6 mM $C_{12}E_8$) was cross-linked with 8 mM glutaraldehyde for 45 min at 22 °C. After the addition of glycine to quench the reaction, the enzyme was denatured with $NaDodSO_4$ and submitted to electrophoresis on a 3.6% polyacrylamide gel. The gel was stained and scanned at 550 nm. An equal amount of enzyme in the membrane-bound form was cross-linked identically and also submitted to electrophoresis. The normal migratory positions of the α-polypeptide and β-polypeptide are indicated. The positions of the new products observed on the gel are indicated by the arrows labeled 1, 2, 3, and 4. The direction of electrophoresis is from left to right with the upper left arrow marking the top of the gels.

Aggregation State of $(Na^+ + K^+)$-ATPase Dissolved in $C_{12}E_8$: Determination by Cross-Linking Assay. For assessment of the aggregation state of this supernatant–$C_{12}E_8$ enzyme, a 50-μL sample, containing 0.5 mg of protein mL^{-1}, <5.6 mM $C_{12}E_8$, 30 mM imidazolium chloride, pH 7.1, 0.1 M KCl, and 10% glycerol, was reacted with 8 mM glutaraldehyde for 60 min at 22 °C. The reaction was quenched with glycine and the sample submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 3A). The normal migration positions of the α- and β-polypeptides are indicated in the figure, and it can be seen that these polypeptides have been completely removed from their respective positions on the gel while new components of lower electrophoretic mobility have appeared.

Several experiments were performed on other samples of supernatant–$C_{12}E_8$ enzyme prepared in the same manner to explore the effects of variations in the cross-linking assay, such as time, temperature, and the concentrations of the protein and the glutaraldehyde, on the patterns observed upon subsequent electrophoresis. No changes in the amounts of the various covalent oligomers were observed with increases in the glutaraldehyde concentration beyond 8 mM (Table I). The time of reaction was varied, halting the cross-linking by addition of a 5-fold molar excess of glycine. It was found that, at 22 °C and 8 mM glutaraldehyde, 45 min of reaction was sufficient to produce a static electrophoretic gel pattern. The

Table I: Effect of Experimental Conditions on Relative Amounts of Covalent Oligomers Observed with Glutaraldehyde Cross-Linking

exptl conditions[b]	integrated area[a]			
	α-β	$(\alpha\text{-}\beta)_2$	$(\alpha\text{-}\beta)_3$	$(\alpha\text{-}\beta)_4$
1.5 mM glutaraldehyde[c]	21	13	3	*f*
6 mM glutaraldehyde[c]	30	22	13	7
8 mM glutaraldehyde[c]	34	27	18	14
12 mM glutaraldehyde[c]	30	27	19	16
16 mM glutaraldehyde[c]	31	28	19	15
10 °C (90 min)[d]	40	30	15	9
15 °C (60 min)[d]	43	29	16	8
22 °C (45 min)[d]	39	30	16	10
30 °C (45 min)[d]	37	28	17	8
0.5 mg mL^{-1} protein[e]	41	33	25	*f*
0.25 mg mL^{-1} protein[e]	46	33	21	*f*
0.125 mg mL^{-1} protein[e]	43	32	14	*f*
0.071 mg mL^{-1} protein[e]	51	35	14	*f*
0.05 mg mL^{-1} protein[e]	47	35	18	*f*

[a] Percent of total staining on the gel. [b] Three separate experiments (*c–e*) are reported. [c] Reaction time was 60 min at 22 °C. [d] Parentheses indicate the time of reaction with 8 mM glutaraldehyde. [e] Reaction time was 60 min at 22 °C and with 8 mM glutaraldehyde. [f] No measurable tetramer.

cross-linking assay, based on these results, is routinely performed for 45 min at 22 °C with a final glutaraldehyde concentration of 8 mM. It was also found that the electrophoretic pattern was independent of temperature within the range tested, 10–30 °C (Table I), an adjustment only being necessary to provide longer incubation periods at temperatures below 22 °C. If the concentration of protein is varied over a factor of 10, the cross-linking pattern is unchanged (Table I). This observation rules out participation of intermolecular collisions in the cross-linking reaction and demonstrates the absence of a concentration-dependent interconversion of the macromolecular complexes present in the solution over the time of the assay. Furthermore, when membrane-bound enzyme is cross-linked under identical conditions, none of the covalent products is capable of entering the $NaDodSO_4$–polyacrylamide gel upon electrophoresis (Figure 3B). This demonstrates that the glutaraldehyde is a sufficiently reactive cross-linking agent to interlink all of the neighbors in a given hydrodynamic particle. Taken together, these observations indicate that the glutaraldehyde cross-linking assay provides an accurate catalog of macromolecular complexes that are present in a given detergent solution prior to the addition of the glutaraldehyde.

The smallest product of the cross-linking reaction, component 1 on Figure 3A, is a covalent heterodimer formed from one α- and one β-polypeptide and therefore was derived from a monomer of the asymmetric unit of the enzyme. Component 1 possesses the same relative electrophoretic mobility (0.44) on 3.6% $NaDodSO_4$–polyacrylamide gels as the α-β heterodimer formed during the reaction of cupric phenanthroline with $(Na^+ + K^+)$-ATPase dispersed in deoxycholate (Craig & Kyte, 1980). Furthermore, component 1, like the cupric phenanthroline product, demonstrates positive staining for carbohydrate with the periodic acid–Schiff stain (Kyte, 1972). Finally, when reaction conditions were varied such that the cross-linking of α and β yielded component 1 as the only significant covalent product observed on gels (Table II), the remaining unreacted amounts of α and β maintained a constant staining ratio to one another (Craig & Kyte, 1980).

The larger components, 2–4, observed on the gel scan in Figure 3A, are covalent products from the higher noncovalent oligomers of the αβ asymmetric unit. The mobilities of the consecutive, noncovalent polymers of bovine serum albumin

Table II: Staining Ratio of α-Polypeptide to β-Polypeptide as a Function of the Extent of Cross-Linking to Exclusively an α-β Heterodimer[a]

cross-linking[b] (%)	α/β
32	2.23
42	2.21
55	2.52
60	2.30
	mean: 2.32 ± 0.12

[a] The addition of 30 mM glycine to the cross-linking mixture as well as short reaction times (2, 5, 15, and 30 min) resulted in the α-β heterodimer being the only major covalent product. [b] Estimated from the amount of the α-polypeptide remaining un-cross-linked on the gel.

Table III: Oligomer Content of Previously Reported Preparations of $(Na^+ + K^+)$-ATPase in Detergent Solutions[a]

detergent	mg of detergent/ mg of protein	integrated area[b]				
		α-β	$(\alpha\text{-}\beta)_2$	$(\alpha\text{-}\beta)_3$	$(\alpha\text{-}\beta)_4$	$(\alpha\text{-}\beta)_5$
Lubrol WX[c]	15	12	24	14	22	22
$C_{12}E_8$[d]	2	25	28	23	20	*f*
$C_{12}E_8$[e]	2.7	54	24	17	4	*f*

[a] Conditions reported earlier were duplicated. [b] Percent of total staining on the gel. [c] Hastings & Reynolds (1979). [d] Esmann et al. (1980). [e] Brotherus et al. (1981). [f] No measurable pentamer.

were determined by denaturing a 100 mg mL^{-1} solution with $NaDodSO_4$ and submitting it to gel electrophoresis under conditions identical with those used for cross-linked enzyme samples. The logarithms of the relative mobilities of the serum albumin polymers were plotted against their equivalent Stokes radii (Fish et al., 1970; Neville, 1971) to yield a linear relationship (Ugel et al., 1971) from which values for the equivalent Stokes radii of the unknown polymers, and hence their apparent polymer length, could be interpolated. The apparent polymer lengths of components 2, 3, and 4 are 1.9, 2.8, and 3.7 times, respectively, the apparent polymer length of component 1, the α-β heterodimer. Furthermore, all of the products of the cross-linking reaction show positive staining for carbohydrate. In summary, these results indicate that components 1, 2, 3, and 4, displayed on the gel scan in Figure 3A, represent the covalent complexes α-β, $(\alpha\text{-}\beta)_2$, $(\alpha\text{-}\beta)_3$, and $(\alpha\text{-}\beta)_4$, respectively, produced during the cross-linking reaction from the noncovalent complexes αβ, $(\alpha\beta)_2$, $(\alpha\beta)_3$, and $(\alpha\beta)_4$ existing in the initial solution and that the areas on the $NaDodSO_4$–polyacrylamide gel (Figure 3A) are directly proportional to their relative weight concentrations in the initial solution.[2]

The aggregation state of $(Na^+ + K^+)$-ATPase dissolved under a variety of conditions in detergents other than $C_{12}E_8$ can also be assessed with the cross-linking assay. The results in Table III represent data taken from scans of cross-linked samples derived from solutions prepared in the same manner as those examined in earlier experiments performed elsewhere (Hastings & Reynolds, 1979; Esmann et al., 1980; Brotherus et al., 1981). It had been claimed that these three preparations are monodisperse solutions containing dimer, dimer, and monomer, respectively. It can be seen, however, that each actually contains a rather complex mixture of different oligomers. The values are presented in Table III as the per-

[2] From here on, monomer, dimer, trimer, and tetramer will refer to the oligomers of the asymmetric unit, αβ, $(\alpha\beta)_2$, $(\alpha\beta)_3$, and $(\alpha\beta)_4$, respectively. The symbol α-β will designate covalent oligomers and αβ, noncovalent.

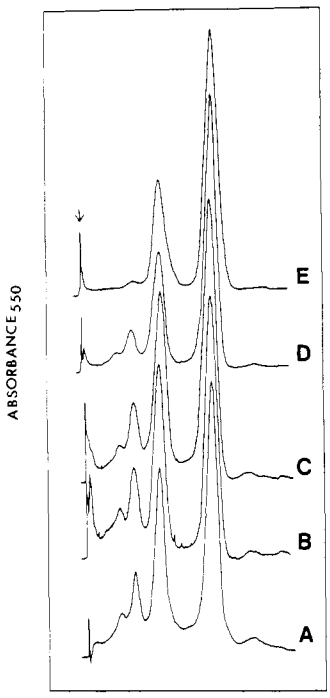

FIGURE 4: Scans of $NaDodSO_4$–polyacrylamide gels of supernatant–$C_{12}E_8$ enzyme treated with increasing concentrations of $C_{12}E_8$. A sample of supernatant–$C_{12}E_8$ enzyme (50 μL of 0.6 mg of protein mL^{-1} and <5.6 mM $C_{12}E_8$) was added to 0.1 mL of a solution containing 20 mM imidazolium chloride, pH 7.1, and $C_{12}E_8$ such that the final additional detergent concentration was 0 (B), 0.5 (C), 5.0 (D), or 50 mM (E). After incubation at 22 °C for 10 min, the samples were cross-linked with glutaraldehyde, denatured, and submitted to electrophoresis. Gel A is a control sample of supernatant–$C_{12}E_8$ enzyme cross-linked without prior dilution. The direction of electrophoresis is from left to right, and the arrow marks the top of the gel.

centage of total staining area on the assay gel although in one preparation (Brotherus et al., 1981) a considerable amount of cross-linked material was unable to enter the 3.6% polyacrylamide gel. In addition, preparations of enzyme dispersed in deoxycholate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, Triton X-100, or the Brij detergents 36T, 56, 58, or 78 all exhibit mixtures of these same oligomers when examined by this quantitative cross-linking assay. In a number of examinations of the effects of these detergents, the relative amounts of each species changed as the type and concentration of the detergent were varied (data not shown), but no monodisperse, enzymatically active complex has yet been observed.

Effect of $C_{12}E_8$ Concentration on the Distribution of Oligomers. Supernatant–$C_{12}E_8$ enzyme was diluted into solutions containing increasing concentrations of detergent. After 10 min, at room temperature, the samples were cross-linked with glutaraldehyde, denatured, and submitted to gel electrophoresis. Scans of stained gels are shown in Figure 4. It can be seen that when supernatant–$C_{12}E_8$ enzyme is diluted into buffer alone there is no significant change in the relative amounts of each oligomer present (Figure 4A,B), with monomer constituting 40–50% of the total protein as stated earlier (Table I). As the detergent:protein ratio is increased, the amount of monomer increases while the level of the higher oligomers decreases (Figure 4C–E). At a higher detergent:protein ratio of 50, monomer comprises over 70% of the total protein while trimer and tetramer have entirely disappeared (Figure 4E). It should be pointed out, however, that even at these high detergent concentrations where ATPase activity has been lost, the dimer has not disappeared entirely and still constitutes nearly 30% of the total protein.

Results very similar to those described above were obtained when membrane-bound enzyme was treated directly with increasing amounts of $C_{12}E_8$ up to a detergent:protein ratio of 55, undispersed membrane was removed by centrifugation, and supernates were assayed by cross-linking (data not shown).

Separation of αβ Oligomers. When a sample of supernatant–$C_{12}E_8$ enzyme is subjected to centrifugation through a 5–20% sucrose gradient containing 16 mM $C_{12}E_8$, the oligomers can be separated on the basis of their sedimentation coefficients. Individual fractions obtained from such a sucrose gradient were assayed for the distribution of oligomers by treatment with 8 mM glutaraldehyde for 60 min at 22 °C. After the reaction was quenched and the polypeptides were denatured, samples of cross-linked fractions were submitted to polyacrylamide gel electrophoresis. Scans of the stained gels allowed a quantitative determination to be made of the amount of each oligomer present in each fraction. Results from several experiments of this type are presented in Figure 5 along with the positions in each of the gradients of several sedimentation velocity standards run concurrently with the supernatant–$C_{12}E_8$ enzyme sample. In Figure 5C, it can be seen that monomer, dimer, trimer, and tetramer have separated from each other when the supernatant–$C_{12}E_8$ enzyme is sedimented through a sucrose gradient containing a large excess of $C_{12}E_8$ (16 mM). A comparison with the standards yields sedimentation coefficients of 6.5 S for monomer, 10 S for dimer, 12 S for trimer, and 14 S for tetramer.[3] Since they can be separated, this result provides independent evidence for the existence of the complexes that the cross-linking assay stated were present in the original supernatant–$C_{12}E_8$ enzyme. Furthermore, when supernatant–$C_{12}E_8$ enzyme is cross-linked prior to centrifugation on the sucrose gradient rather than after centrifugation, the distribution and relative amounts of the various complexes are very similar (data not shown). These results, taken together, suggest that the complexes observed in supernatant–$C_{12}E_8$ enzyme are discrete and stable entities.

When the $C_{12}E_8$ concentration in the gradient is decreased, the molecular complexes are less well resolved (Figure 5B). This is due to alterations in the sedimentation coefficients of monomer, dimer, and trimer, which are all shifted to within the range of 6–10 S. Upon a further decrease in the concentration of detergent in the gradient (Figure 5A), the apparent sedimentation coefficients of monomer, dimer, and trimer become indistinguishable from each other (7 ± 1 S).

Aggregation State during Enzyme Turnover. A sample of supernatant–$C_{12}E_8$ enzyme containing monomer, dimer, trimer, and tetramer was submitted to the cross-linking assay after addition to the enzymatic assay medium: 3 mM Mg-ATP, 100 mM NaCl, 20 mM KCl, and 30 mM imidazolium chloride, pH 7.1. The sample was submitted to electrophoresis, and a scan of the stained gel is shown in Figure 6B. It can be seen that under the same conditions where the enzyme was actively turning over in a companion tube (116 μmol of P_i mg^{-1} h^{-1} at 22 °C) the majority of the protein had aggregated to oligomers greater in size than that of a tetramer so that the majority (70%) of the products of the cross-linking assay could not enter the 3.6% polyacrylamide gel. As a control, an identical sample of supernatant–$C_{12}E_8$ enzyme was diluted into buffer alone, and little aggregation occurred (Table I, Figure 6A). Therefore, even though the enzyme is dispersed to small

[3] A small quantity of an additional complex is observed that sediments between monomer and dimer. This complex is probably a slightly altered form of dimer since the cross-linked products of both the dimer and the undefined complex possess similar electrophoretic mobilities on $NaDodSO_4$ gels (data not shown).

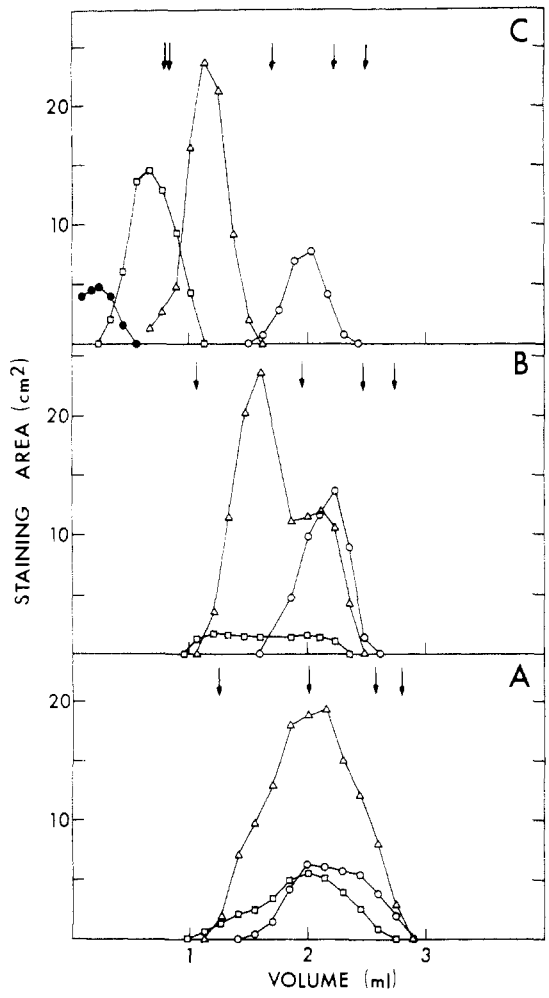

FIGURE 5: Separation of supernatant–$C_{12}E_8$ enzyme on linear sucrose gradients containing $C_{12}E_8$. Samples (100 μL) of $(Na^+ + K^+)$-ATPase dissolved in solutions of $C_{12}E_8$ [7.4 μmol of $C_{12}E_8$ (mg of protein)$^{-1}$] were clarified by centrifugation and submitted to a second centrifugation through 5–20% linear sucrose gradients containing $C_{12}E_8$ as noted. Fractions collected from the gradients were analyzed for the presence of the various complexes, monomer, dimer, trimer, and tetramer, with the glutaraldehyde cross-linking assay. Stained $NaDodSO_4$–polyacrylamide gels of each of the cross-linked fractions were scanned in order to quantify the amount of each complex present in the individual fractions. Indicated in the graphs is the absolute staining area of the covalent complexes α-β (○), $(\alpha$-$\beta)_2$ (△), $(\alpha$-$\beta)_3$ (□), and $(\alpha$-$\beta)_4$ (●) present in each fraction assayed. The amount of protein added to each gradient, the $C_{12}E_8$ concentration in each gradient, and the time of each run, respectively, were the following: (A) 0.58 mg of $(Na^+ + K^+)$-ATPase, 2 mM $C_{12}E_8$, 5.5 h; (B) 0.26 mg of $(Na^+ + K^+)$-ATPase, 6 mM $C_{12}E_8$, 6 h; (C) 0.16 mg of $(Na^+ + K^+)$-ATPase, 16 mM $C_{12}E_8$, 6.5 h. The recovered volume from each gradient was 3.7 mL, and the bottoms are to the left. The positions of sedimentation standards for each of the three experiments were determined from companion gradients and are indicated on the graphs by the upper arrows. The standards used were bovine serum albumin (4.7 S), human transferrin (6.1 S), lactate dehydrogenase (7.2 S), and aspartate transcarbamylase (11.7 S) in that order from right to left. Additionally, bovine catalase (11.3 S) was used as a standard for the gradient shown in (C).

oligomers by the detergent, it reaggregates upon enzymatic assay.

Discussion

The glutaraldehyde cross-linking assay accurately determines the aggregation state of $(Na^+ + K^+)$-ATPase as it exists in detergent solution. Conditions can be found in which the reaction produces a static, reproducible pattern of covalent products, which can be visualized on $NaDodSO_4$–polyacrylamide gels, and increases in the concentration of glutaraldehyde or incubation time, or variation in the protein concentration, produce no additional changes in the electrophoretic pattern (Table I). Furthermore, when the un-cross-linked, supernatant enzyme preparation is subjected to centrifugation on a sucrose gradient, monomers, dimers, trimers, and tetramers of the asymmetric unit can be separated from one another, and each gives the expected, covalent product when fractions from the gradient are assayed by cross-linking (Figure 5C). This demonstrates that predictions, based on conclusions from the cross-linking assay, are fulfilled in an experiment. Finally, further evidence for the validity of this assay is the observation that membrane-bound enzyme is completely cross-linked into complexes of such large size that they are incapable of entering a 3.6% $NaDodSO_4$–polyacrylamide gel upon electrophoresis. Presumably, these large structures are formed from random intermolecular collisions of molecules confined at very high concentration within the two dimensions of the bilayer (Kyte, 1981).

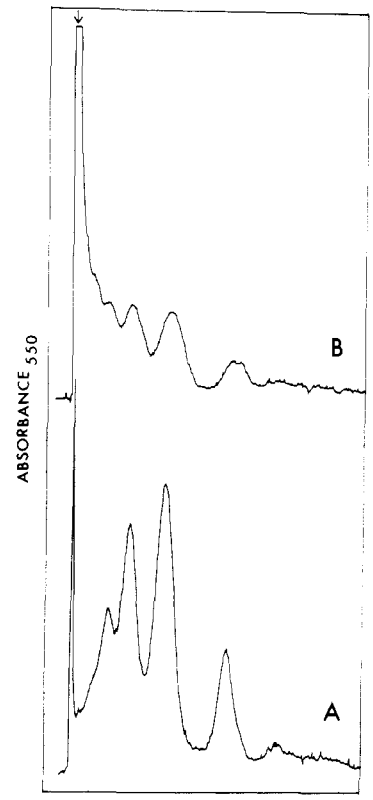

FIGURE 6: Scans of $NaDodSO_4$–polyacrylamide gels of supernatant–$C_{12}E_8$ enzyme cross-linked in the presence (B) or absence (A) of assay substrates. Two samples of supernatant–$C_{12}E_8$ enzyme (50 μL of 0.2 mg of protein mL^{-1}) were diluted either into buffer alone or into assay medium, respectively. After a 5-min incubation, the samples were cross-linked and submitted to $NaDodSO_4$–polyacrylamide gel electrophoresis. The direction of electrophoresis is from left to right with the arrow marking the tops of the gels.

In a recent report, the usefulness of a glutaraldehyde cross-linking assay was demonstrated convincingly in experiments on solutions of a soluble protein, lactate dehydrogenase (Hermann et al., 1981). The results presented there permitted direct comparisons to be made between rates of enzymatic reactivation and rates of oligomer formation. Furthermore, the requisite tests to prove the assay valid were performed as rigorously then as they are here, and the conclusions reached were the same, namely, that the quantitative cross-linking assay can provide accurate, sensitive, and reliable assessments of the distribution of the noncovalent oligomeric structures in a given sample. In the present study, lower glutaraldehyde concentrations were used to reduce excess reagent and avoid elaborate

quenching procedures; nonetheless, the results and conclusions are as valid.

Several experimental observations address the question of whether or not interconversions can occur between the noncovalent protein complexes formed when $(Na^+ + K^+)$-ATPase is dispersed in a nonionic detergent solution. As indicated in Table I, protein concentration can be varied over a 10-fold range without affecting the relative concentrations of the complexes. Furthermore, in Figure 4, it can be seen that it is only variation in the detergent:protein ratio that brings about a conversion of one complex to another. Hence, while there are changes effected by variations in the detergent:protein ratio, there is no evidence that interconversions exist that are dependent on protein concentration. No evidence for time-dependent changes was ever observed either. The 10 min between the addition of enzyme into excess detergent and the initiation of the cross-linking reaction, as in the experiment in Figure 4, is sufficient for the system to reach an unchanging composition. All subsequent cross-linking experiments performed in this laboratory have supported this conclusion. Lastly, the complexes, monomer, dimer, and trimer, can be separated on sucrose gradients containing detergent when they are submitted to centrifugation for 6 h (Figure 5). From this latter result alone, it can be concluded that the system of noncovalent complexes added to the gradient has reached a state that does not change in response to variations in the concentration of any of the species over the time of the experiment. Therefore, the set of complexes formed in the nonionic detergent solution does not appear to be altered by changes in protein concentration or variations in time. Although this is in itself interesting, the question of whether or not any time-dependent or concentration-dependent changes could be observed under other circumstances neither is relevant to the conclusions now to be drawn nor was it explored in detail.

Several earlier experiments, which address the question of the minimum functional unit of $(Na^+ + K^+)$-ATPase, can be evaluated more critically in light of the observations presented here. Efforts to determine whether the native, active $(Na^+ + K^+)$-ATPase is a monomer or a dimer have generally involved two approaches. Techniques such as radiation inactivation (Kepner & Macey, 1968; Ellory et al., 1979), ligand binding stoichiometry (Perrone et al., 1975; Jørgensen, 1977; Petersen et al., 1978), and chemical cross-linking (Kyte, 1975; Giotta, 1976; Liang & Winter, 1977) have been employed to determine either protein mass $(\text{active site})^{-1}$ or subunit associations in membrane-bound preparations of the enzyme. The difficulties and inadequacies of these methods have been discussed previously (Craig & Kyte, 1980; Kyte, 1981), and it is clear that, although these approaches have been provocative, they are incapable of answering the question.

On the other hand, the enzyme can be released from the membrane by the bile salts, deoxycholate and cholate (Kyte, 1971; Goldin, 1977), and several nonionic detergents (Hokin et al., 1973; Hastings & Reynolds, 1979; Esmann et al., 1980; Brotherus et al., 1981). With certain detergents, there are rather narrow limits within which unstable but soluble forms of $(Na^+ + K^+)$-ATPase can be produced (Kyte, 1971; Brotherus et al., 1979). Nevertheless, examples of enzymatically stable, soluble preparations formed during treatment of membranes with several nonionic detergents have recently been described (Hokin et al., 1973; Hastings & Reynolds, 1979; Esmann et al., 1980; Brotherus et al., 1981). It is with these latter detergents that the formation of apparently discrete, multimeric complexes of $(Na^+ + K^+)$-ATPase, which are said to possess apparent protein molecular weights of 380000 ± 21000 in Lubrol WX (Hastings & Reynolds, 1979) and 256000 ± 23000 in $C_{12}E_8$ (Esmann et al., 1980), has been reported. These values were calculated from hydrodynamic properties of the complexes of protein, detergent, carbohydrate, and phospholipid present in these solutions and are significantly larger than the 140000–180000 molecular weight expected for a monodisperse monomer. On the other hand, the preparation, in $C_{12}E_8$, of a complex, capable of regaining activity, with an apparent protein molecular weight of 170000 ± 9000, estimated from hydrodynamic properties, has also been described (Brotherus et al., 1981).

An examination of the results presented in Table III suggests that the differences in the protein molecular weights calculated from the hydrodynamic properties of preparations of $(Na^+ + K^+)$-ATPase dissolved in the various detergents do not result from differences in the type of oligomer, monomer, or dimer actually present in the solution. On the contrary, they presumably reflect differences in the weight-average molecular weights of the complicated mixtures of the various complexes formed under these different conditions. Single, discrete, monodisperse complexes are never present regardless of the detergent concentration (Figure 4), and it must be assumed that the techniques of ultracentrifugation commonly employed are incapable of detecting this fact. On the other hand, the glutaraldehyde cross-linking assay can clearly resolve each oligomer of the enzyme, can detect all complexes present, and can display the proportion of each (Hermann et al., 1981).

Another question, which has not been adequately addressed, is what the quaternary structure of the soluble $(Na^+ + K^+)$-ATPase is when it is actually turning over in an enzymatic assay. A chemical cross-linking assay has advantages that permit it to make such a determination. As Figure 6 demonstrates, it is possible to assess the aggregation state of the enzyme in the presence of substrates provided none are strong nucleophiles. In this manner, the aggregation state of functioning complexes can be determined, something all previous experiments have failed to do. The results suggest that rather striking changes in the relative proportions of the various oligomers occur upon addition of substrates, and they bring into question (Kyte, 1981) earlier claims that active-transport enzymes can function as monomers (Møller et al., 1980; Brotherus et al., 1981). It can be concluded that the issue of whether the functional unit of $(Na^+ + K^+)$-ATPase is a monomer or a dimer has not yet been resolved.

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References

Brotherus, J. R., Jost, P. C., Griffith, O. H., & Hokin, L. E. (1979) *Biochemistry 18*, 5043–5050.

Brotherus, J. R., Møller, J. V., & Jørgensen, P. L. (1981) *Biochem. Biophys. Res. Commun. 100*, 146–154.

Clarke, S. (1975) *J. Biol. Chem. 250*, 5459–5469.

Craig, W. S., & Kyte, J. (1980) *J. Biol. Chem. 255*, 6262–6269.

Ellory, J. C., Green, J. R., Jarvis, S. M., & Young, J. D. (1979) *J. Physiol. (London) 295*, 10–11P.

Esmann, M., Christiansen, C., Karlsson, K., Hansson, G. C., & Skou, J. C. (1980) *Biochim. Biophys. Acta 603*, 1–12.

Fish, W. W., Reynolds, J. A., & Tanford, C. (1970) *J. Biol. Chem. 245*, 5166–5168.

Giotta, G. (1976) *J. Biol. Chem. 251*, 1247–1252.

Goldin, S. M. (1977) *J. Biol. Chem. 252*, 5630–5642.

Hastings, D. F., & Reynolds, J. A. (1979) *Biochemistry 18*, 817–821.
Helenius, A., McCaslin, D. R., Fries, E., & Tanford, C. (1979) *Methods Enzymol. 56*, 734–749.
Hermann, R., Jaenicke, R., & Rudolph, R. (1981) *Biochemistry 20*, 5195–5201.
Hokin, L. E., Dahl, J. L., Deupree, J. D., Dixon, J. F., Hackney, J. F., & Perdue, J. F. (1973) *J. Biol. Chem. 248*, 2593–2605.
Jørgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36–52.
Jørgensen, P. L. (1977) *Biochim. Biophys. Acta 466*, 97–108.
Kepner, G. R., & Macey, R. I. (1968) *Biochim. Biophys. Acta 163*, 188–203.
Kyte, J. (1971) *J. Biol. Chem. 246*, 4157–4165.
Kyte, J. (1972) *J. Biol. Chem. 247*, 7642–7649.
Kyte, J. (1975) *J. Biol. Chem. 250*, 7443–7449.
Kyte, J. (1981) *Nature (London) 292*, 201–204.
Liang, S.-M., & Winter, C. G. (1977) *J. Biol. Chem. 252*, 8278–8284.
Møller, J. V., Lind, K. E., & Andersen, J. P. (1980) *J. Biol. Chem. 255*, 1912–1920.
Munson, K. (1981) *J. Biol. Chem. 256*, 3223–3230.
Neville, D. M., Jr. (1971) *J. Biol. Chem. 246*, 6328–6334.
Perrone, J. R., Hackney, J. F., Dixon, J. F., & Hokin, L. E. (1975) *J. Biol. Chem. 250*, 4178–4184.
Petersen, G. L. (1978) *Anal. Biochem. 84*, 164–172.
Petersen, G. L., & Hokin, L. E. (1981) *J. Biol. Chem. 256*, 3751–3761.
Petersen, G. L., Ewing, R. D., Hootman, S. R., & Conte, F. P. (1978) *J. Biol. Chem. 253*, 4762–4770.
Ugel, A. R., Chrambach, A., & Rodbard, D. (1971) *Anal. Biochem. 43*, 410–426.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.
Winter, C. G., & Moss, A. J., Jr. (1979) in *Na,K-ATPase: Structure and Kinetics* (Skou, J. C., & Nørby, J. G., Eds.) pp 25–32, Academic Press, New York.

Increased Level of Translatable Collagenase Messenger Ribonucleic Acid in Rabbit Synovial Fibroblasts Treated with Phorbol Myristate Acetate or Crystals of Monosodium Urate Monohydrate†

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ABSTRACT: We studied mechanisms governing production of the neutral proteinase collagenase by synovial cells. We used a model system of monolayer cultures of rabbit synovial fibroblasts stimulated to produce collagenase by treatment with phorbol myristate acetate or crystals of monosodium urate monohydrate. mRNAs from these and untreated cells were translated in a wheat germ cell-free system. Collagenase was not present in the culture medium or in the in vitro translation products of mRNA from untreated cells but was present in both the medium and translation products of stimulated cells, as analyzed by gel electrophoresis and immunoprecipitation with monospecific antibody. Induction of collagenase was prevented by treatment of the cells with α-amanitin (2 μg/mL), an inhibitor of mRNA synthesis. We have concluded that the induction of collagenase synthesis by either phorbol myristate acetate or urate crystals is due to an increased level of translatable mRNA.

Synovial cells taken from patients with rheumatoid arthritis are known to synthesize and secrete large quantities of the neutral proteinase collagenase. The importance of this enzyme in mediating collagenolysis in rheumatoid disease is well established (Harris & Krane, 1974; Harris, 1978, 1981), but mechanisms controlling collagenase production are unclear.

We have developed a model system for rheumatoid synovium in which monolayer cultures of rabbit synovial fibroblasts can be stimulated experimentally to produce large amounts of collagenase. Untreated cultures secrete negligible amounts of this enzyme, but treatments with membrane active agents such as poly(ethylene glycol) (Brinckerhoff & Harris, 1978) or cytochalasin B (Harris et al., 1975), phagocytosis of insoluble debris (Werb & Reynolds, 1975), and a factor secreted by mononuclear cells (Dayer et al., 1976, 1978) are all capable of inducing collagenase synthesis. Two additional and potent inducers of collagenase are crystals of monosodium urate monohydrate (McMillan et al., 1981) and the tumor promoter phorbol myristate acetate (PMA) (Brinckerhoff et al., 1979).

No mechanism for collagenase induction by any of these stimuli has been postulated, although we previously measured a series of intracellular events occurring after addition of PMA (Brinckerhoff et al., 1979). In response to PMA, intracellular cyclic AMP levels increased temporarily at 10 min, followed by a transient decrease in DNA synthesis (maximum effect at 9–12 h), a rise in the PGE_2 level in culture medium (starting at 12–24 h), and a rise in collagenase activity that was detectable in the culture medium at about 24 h.

Since collagenase is not stored in cells after synthesis (Valle & Bauer, 1979), the 24-h lag period appeared to represent the time during which active cytoplasmic collagenase mRNA was produced. This could be the result of immediate synthesis of collagenase mRNA (in response to PMA or urate crystals) that remained in a translationally inactive form until 20–24 h, or it may represent the time needed for functionally active

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