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Isolation and Properties of a Chloroplast Coupling Factor and Heat-Activated Adenosine Triphosphatase*

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ABSTRACT: The purification to homogeneity of a coupling factor (CF_1) from spinach chloroplasts is described. Conversion of the coupling factor, a latent ATPase, to a manifest ATPase by mild heat treatment in the presence of ATP yields a protein which is identical with CF_1 with regard to (a) electrophoretic mobility on polyacrylamide gel, (b) sedimentation patterns in the analytical ultracentrifuge, and (c) amino acid composition. The molecular weight of the native coupling factor and the heat-activated ATPase determined by high-speed sedimentation to equilibrium was found to be 325,000. In the presence of 5 M guanidine-HCl the apparent molecular weight was found to be 62,000 by high-speed sedimentation to equilibrium. From amino acid analysis a minimal molecular weight of 28,000 was calculated.

Studies on a coupling factor from chloroplasts (Avron, 1963; Vambutas and Racker, 1965) have revealed considerable similarities to a coupling factor isolated from beef heart mitochondria (Pullman *et al.*, 1960). Both proteins are cold-labile in solution (Penefsky *et al.*, 1960; McCarty and Racker, 1966) and show similar structures in the electron microscope (Vambutas and Racker, 1965). The soluble mitochondrial coupling factor has ATPase activity (Penefsky *et al.*, 1960), whereas, the chloroplast factor is a latent ATPase and exhibits hydrolytic activity only after treatment with trypsin or heat (Vambutas and Racker, 1965). Pullman and Monroy (1963) have isolated a trypsin-sensitive protein from mitochondria which is a specific inhibitor of mitochondrial ATPase. This inhibitor forms a soluble complex with mitochondrial ATPase which has no hydrolytic activity, but serves as a coupling factor. The masked ATPase activity of this complex can be reactivated by heat treatment.

These findings raised the possibility that CF_1[1] may represent a firmly associated ATPase–inhibitor complex. However, efforts to isolate an inhibitor from CF_1 or from chloroplasts by a procedure similar to that of Pullman and Monroy were unsuccessful (Farron, 1969). Efforts were therefore made to establish whether any part of the coupling factor dissociates irreversibly during heating. In order to do this without ambiguity it was imperative to work with a homogeneous preparation. In the present paper the purification of the coupling factor to homogeneity is described, together with studies of some physicochemical and chemical properties of the protein before and after heat activation. The data indicate that the conversion of latent into manifest ATPase by heat treatment does not entail gross changes in conformation, in the state of aggregation, in electrophoretic mobility, or in amino acid composition.

Experimental Procedure

Materials. Gd·HCl (Ultra Pure) was purchased from Mann Research Laboratories and was used without further purification.

Methods. Amino acid analyses were carried out on a Beckman Spinco Model 120C amino acid analyzer by the method of Spackman *et al.* (1958). Disc gel electrophoresis was performed according to Ornstein and Davis (1964); instead of mixing the sample with spacer gel the sample was

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: chloroplast coupling factor (CF_1) and guanidine hydrochloride (Gd·HCl).

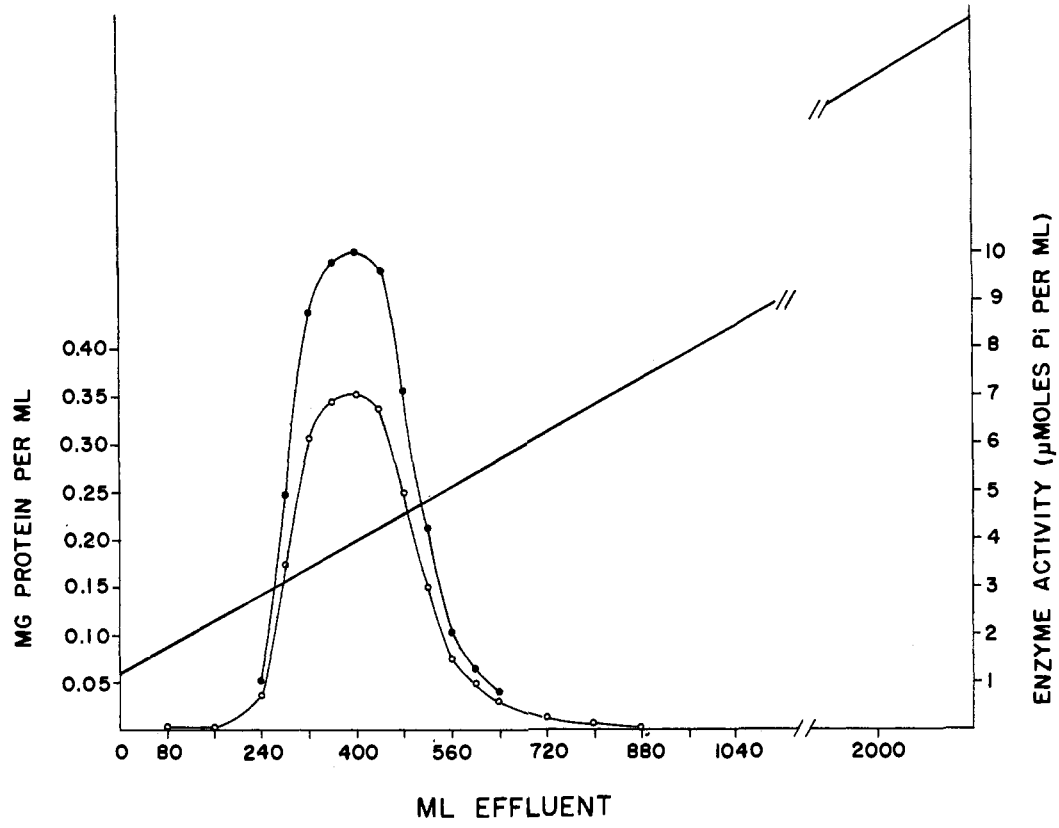

FIGURE 1: Chromatography of CF_1 on hydroxylapatite. The column was prepared and developed as described in the text: (—O—) mg of protein per ml; (—●—) ATPase activity; (——) phosphate gradient between 0.010 M P_i, pH 6.9, and 0.15 M P_i, pH 7.9.

introduced in a layer of buffer containing 20% sucrose, between the reservoir buffer and the concentration gel.

Ultracentrifugal studies were performed in a Spinco Model E analytical ultracentrifuge equipped with schlieren and interference optical systems and the RTIC temperature control system. All determinations were carried out at 25°. Samples were equilibrated with 0.075 M phosphate buffer, pH 7.1, containing 2 mM EDTA and 4 mM ATP, on a 1 × 12 cm column of Sephadex G-50. For low-speed experiments the AN-J rotor was used, for high-speed experiments the AN-D rotor was used. For the analyses in guanidine an 8 M solution was brought to pH 7.1 by addition of NaOH and was added to the protein sample to give a final concentration of 5 M Gd·HCl. The reference solution was prepared in the same way, except that buffer (column effluent) was used instead of the protein solution. The photographic plates were read with the aid of a Gaertner two-dimensional microcomparator. Viscosity measurements were made with a Cannon-Manning semimicroviscometer from Cannon Instrument Co., State College, Pa. Protein concentrations were expressed as milligrams of dry weight per milliliter. The extinction coefficient was found to be $\epsilon_{280}^{1\%}$ 5.4.

Ca^{2+}–ATPase activity after activation by trypsin was assayed as described by Vambutas and Racker (1965). Inorganic phosphate was determined by the method of Lohmann and Jendrassik (1926).

Circular dichroism studies were kindly performed by Mr. S. Schullery, on a Jasco optical rotatory dispersion recorder, Model ORD/UV-5.

Definition of Unit and Specific Activity. A unit of ATPase is defined as the amount of enzyme required to catalyze the hydrolysis of 1 μmole of ATP per min under the specified assay conditions. Specific activity is expressed as units per milligram of dry weight of protein.

Results and Discussion

Purification of the Coupling Factor to Homogeneity. The purification procedure which gave a homogeneous preparation of the coupling factor is summarized in Table I. The first two steps were carried out essentially as described by Vambutas and Racker (1965), except that the preparation was scaled up about sixfold, as follows: chloroplasts, derived from 3 kg of washed, deveined spinach leaves, were prepared by the procedure of Jagendorf and Avron (1958). The chloroplast suspension was adjusted to contain approximately 3 mg of chlorophyll per ml and was added slowly to 16 volumes of acetone precooled to −10° with rapid stirring. The acetone was then decanted, the residue was spread out on a glass plate and dried in a stream of cold air. The residue was extracted three times with 50 mM Tris-Cl buffer, pH 8.0, containing 2 mM EDTA and 4 mM ATP for 20 min at room temperature and centrifuged at 20,000*g* for 10 min. To the supernatant solution (crude extract) 12 g of solid ammonium sulfate per 100 ml of solution was added at 0° with slow stirring. It was found that it was best to interrupt the preparation at this point, and to store the solution containing the first addition of ammonium sulfate in the refrig-

TABLE I: Purification of Coupling Factor 1 from Chloroplast.

Fraction	Volume (ml)	Total Protein (mg)	Total Units	Sp Act.	Yield (%)
1. Crude extract	295	2120	11,020	5.2	100
2. Ammonium sulfate precipitate	31	715	10,150	14.2	92
3. DEAE-Sephadex eluate after precipitation and desalting	22.4	264	7,480	28.3	68
4. Hydroxylapatite eluate	400	145	4,120	28.4	37

TABLE II: Amino Acid Composition of CF_1 and F_1. Analyses Were Carried Out on Desalted, Lyophilized Samples in 6 N HCl at 110° in Evacuated Sealed Tubes for the Times Indicated.

	Number of Residues per Cysteic Acid[b]					
	CF_1					F_1
Amino Acid[a]	24 hr	40 hr	71 hr	20 hr[c]	Int No.	24 hr
Lysine	10.9	10.79	10.43	10.49	11	14.1
Histidine	1.98	1.83	1.63	1.23	2	3.8
Arginine	15.55	15.51	14.86	14.90	16	14.9
Cysteic acid				0.95	1	
Aspartic acid	17.61	18.24	18.60	17.09	19	18.5
Threonine	15.80	14.67	13.30	16.40	18	12.4
Serine	13.20	10.95	8.87	13.55	15	11.7
Glutamic acid	34.70	34.51	34.90	34.19	35	28.0
Proline	9.85	10.55	9.05	9.96	10	10.2
Glycine	20.59	21.44	20.98	20.56	21	21.4
Alanine	23.00	23.00	23.00	21.85	23	25.0
Valine	17.08	17.90	17.81	17.97	18	18.3
Methionine	6.1	5.46	5.46		7	4.4
Methionine sulfoxide				6.4		
Isoleucine	15.02	16.0	16.67	16.0	17	15.2
Leucine	23.34	23.7	23.6	24.0	24	20.3
Tyrosine	6.7	6.5	6.35		7	6.7
Phenylalanine	6.9	6.65	6.47	6.47	7	6.8

[a] Assays for the presence of tryptophan in CF_1 were carried out by Dr. Gordon H. Ellis according to the method of Hopkins and Cole (Hawk *et al.*, 1947) and were found to be negative. [b] All values were normalized to alanine = 23.00 after the constancy of the ratio alanine:cysteic acid had been established and that the recovery of alanine is unaffected by performic acid oxidation. [c] Analysis was carried out as described above except that the sample was oxidized with performic acid prior to hydrolysis, as described in the text.

erator overnight. The next day the precipitate was collected by centrifugation and discarded and 13.8 g of solid ammonium sulfate per 100 ml was added to the supernatant solution at 0°. The precipitate, containing the enzyme, was collected by centrifugation and dissolved at room temperature in a minimal volume of 20 mM Tris-SO_4 buffer, pH 7.1, containing 2 mM EDTA. All subsequent operations were carried out at room temperature. The solution was clarified by centrifugation at 20,000*g* for 20 min and was then diluted to contain no more than 80 mM NH_3 assayed with Nessler's reagent. This solution was then applied to a 250-ml column of DEAE-Sephadex A-50 equilibrated with 20 mM Tris-SO_4 buffer, pH 7.1, containing 2 mM EDTA, 1 mM ATP, and 80 mM ammonium sulfate. The enzyme was eluted with a linear gradient with respect to ammonium sulfate generated by having 1 l. of the above buffer in the mixing chamber and 1 l. of 20 mM Tris-SO_4 buffer, pH 7.1, containing 2 mM EDTA, 1 mM ATP, and 0.31 M ammonium sulfate in the reservoir. This procedure was described by Bennun and Racker (1969) except that the buffer used by these authors did not contain ATP. This omission was shown to cause an inactivation of the ATPase which was reversed by ATP. The active fractions eluted from the DEAE-Sephadex column were pooled, precipitated with an equal volume of saturated ammonium sulfate brought to pH 7.1 with concentrated ammonia, redissolved in a minimal volume of 0.01 M phos-

TABLE III: Amino Acid Composition of the Native Coupling Factor and the Heat-Activated ATPase after Performic Acid Oxidation. Samples Were Hydrolyzed for 20 hr at 110° in 6 N HCl in Evacuated Sealed Tubes.

Amino Acid	Number of Residues per Cysteic Acid	
	Native Factor	ATPase
Cysteic acid	1.0	1.0
Aspartic acid	17.19	17.09
Threonine	15.43	16.0
Serine	13.20	13.35
Glutamic acid	34.68	34.17
Proline	10.12	10.31
Glycine	21.1	21.0
Alanine	23.0	23.2
Valine	13.5	12.8
Methionine sulfoxide	7.0	7.0
Isoleucine	14.0	13.7
Leucine	23.9	23.7
Tyrosine		Trace
Phenylalanine	5.1	5.1

phate buffer, pH 6.9, and passed through a 1.3 × 50 cm column of Sephadex G-50 equilibrated with the same buffer. The eluate, containing approximately 260 mg of protein, was applied to a 2.2 × 25 cm column of hydroxylapatite (Levin, 1962) equilibrated with 0.01 M phosphate buffer, pH 6.9, containing 1 mM ATP. The column was developed at a flow rate of 10 ml/hr with a linear gradient with respect to both pH and phosphate concentration, generated by having 1 l. of 0.01 M phosphate buffer, pH 6.9, containing 1 mM ATP in the mixing chamber, and 0.15 M phosphate buffer, pH 7.9, containing 1 mM ATP in the reservoir. The active fractions emerged between 0.025 and 0.05 M phosphate and the specific activity of the ATPase was constant over the entire peak. The elution profile is shown in Figure 1. Fractions taken across the entire peak gave one single band on polyacrylamide gel when analyzed immediately after emergence from the column, but two additional faint, slower moving bands were observed on samples that had been standing for as little as 2 days at room temperature or had been stored in 2 M ammonium sulfate at 0°. Although no increase in specific activity resulted from this last step it enabled us to demonstrate that a homogeneous preparation of the protein could be obtained.

Preparation of Heat-Activated ATPase. An aliquot of coupling factor, containing 2 to 5 mg of protein, which had been stored at 0° in 2 M ammonium sulfate, pH 7.5, 2 mM EDTA, and 2 mM ATP was centrifuged, redissolved in a minimal volume of 40 mM Tris-HCl, pH 7.8, containing 2 mM EDTA, and passed through a 1 × 10 cm column of Sephadex G-50 equilibrated with the same buffer. The fractions containing protein were located by their absorption at 280 mμ. The protein concentration was adjusted to be within 0.8–1.8 mg/ml and supplemented with 0.2 M ATP, pH 7.8, to give a final concentration of 40 mM ATP. The solution was then submerged in a water bath kept at 62° for 4 min.

Comparison of the Coupling Factor and the Heat-Activated ATPase. AMINO ACID ANALYSIS. Samples of the desalted protein were hydrolyzed in 6 N HCl at 110° in evacuated sealed tubes for 24, 40, and 71 hr, respectively, and for 20 hr after performic acid oxidation (Moore, 1963) and duplicates of each hydrolysate were analyzed (Spackman *et al.*, 1958). The results are given in Table II. Since the recoveries of some amino acids vary as a function of hydrolysis time, the values listed in the sixth column of the table represent maxima obtained by extrapolation to zero time for amino acids which are destroyed and to maximum yield for amino acids which are released slowly on acid hydrolysis. It seemed of interest to include (last column) values for a 24-hr hydrolysate of the mitochondrial F_1 since these data indicate another similarity between the two proteins.

To establish whether any part of the protein was released upon heat activation, 5 mg of CF_1 were heated at 62°, as described above, and the entire reaction mixture was applied to a 0.9 × 60 cm column of Sephadex G-75. The ATPase emerged immediately after the void volume; all fractions emerging after those containing the ATPase up to and including the salt volume were pooled, lyophilized, oxidized with performic acid, and hydrolyzed in identical fashion as the coupling factor and the ATPase. Amino acid analyses from this experiment are shown in Table III together with an amino acid analysis of CF_1 carried out under identical conditions. No significant difference between the two proteins could be detected. Moreover, the fractions collected after the ATPase and including the salt volume contained no amino acids.

ELECTROPHORESIS ON POLYACRYLAMIDE GEL. The native coupling factor and the heat-activated ATPase exhibited identical mobilities on polyacrylamide gel at pH 7.4 and pH 8.6.

STUDIES IN THE ANALYTICAL ULTRACENTRIFUGE. a. Sedimentation analysis. The sedimentation coefficient of the native coupling factor was determined at protein concentrations from 7.1 to 0.7 mg per ml, and that of the heat-activated ATPase from 3.6 to 0.7 mg per ml using schlieren optics. The analyses at concentrations from 7.1 to 3.25 mg per ml were made in 12-mm double sector cells, lower concentrations were analyzed in 30-mm cells or using ultraviolet optics and reading the half-height of the boundary. In the latter case the buffer contained 0.06 mM ATP instead of 4 mM. This low ATP concentration appeared sufficient to stabilize the enzyme during the short time required for sedimentation analysis. The dependence of the sedimentation coefficient on concentration is shown in Figure 2; it is apparent that no concentration dependent change in the state of aggregation of the enzyme takes place. The value extrapolated to zero protein concentration and corrected for the viscosity of the solvent relative to the viscosity of water at 20° was found to be $s^0_{w,20}$ = 13.8 S for both the coupling factor and the ATPase. The partial specific volume, $\bar{v}$, was determined experimentally by pycnometry using a pycnometer of 2-ml capacity, and calculated from the amino acid composition according to the procedure of Cohn and Edsall (1943) and found to be 0.73 (7) by the former and 0.737 by the latter method. A value of 0.737 was used in all calculations of molecular weights, including data obtained in 5 M Gd·HCl which appears to be a valid procedure (Reithel *et al.*, 1964).

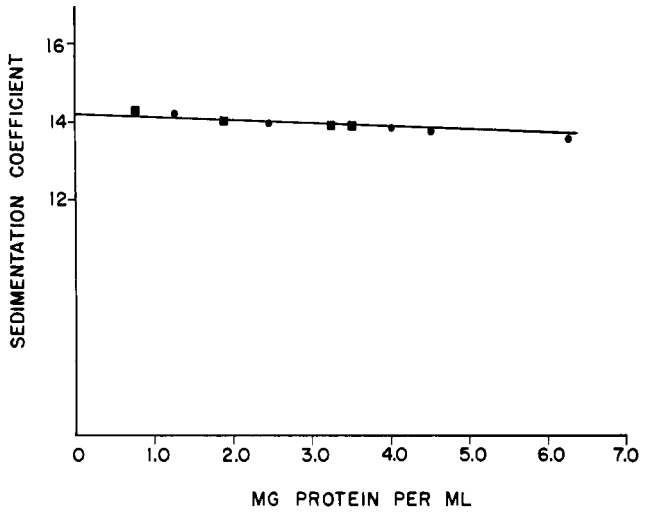

FIGURE 2: Dependence of the sedimentation coefficient of CF_1 and ATPase on protein concentration: (—■—) ATPase; (—●—) CF_1.

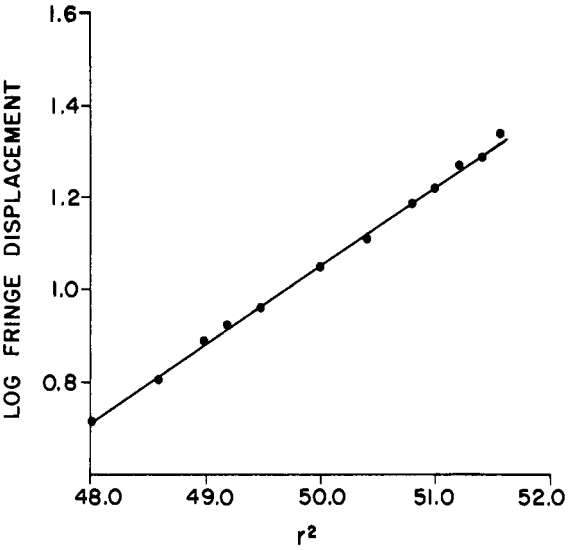

FIGURE 3: Dependence of log c on r^2 during high-speed sedimentation to equilibrium. The rotor speed was 10,440 rpm. The protein concentration was 0.36 mg/ml. The temperature was 23°. Equilibrium was reached after 21 hr.

b. Molecular weight determination. High-speed sedimentation to equilibrium was carried out according to the method of Yphantis (1964) at speeds of 10,000 and 14,000 rpm for both proteins at concentrations of 0.36 mg/ml using interference optics. The dependence of log c on r^2 is shown in Figure 3; on the ordinate are plotted values of log of fringe displacements greater than 100 μ. The dependence was found to be linear throughout 90% of the liquid column being analyzed. The molecular weight determined by this method was found to be 325,000 with a standard deviation of ±600 (three separate experiments). Determinations were also made at low-speed sedimentation to equilibrium at 4400 rpm and protein concentrations of 0.6 mg/ml using the method of LaBar (1965) to ascertain the protein concentration at the meniscus. The molecular weight found by this method was 358,000 with a standard deviation of ±3100 (five different experiments). The discrepancy between the two methods can most reasonably be accounted for by the fact, mentioned above, that samples that had been stored for as short periods as 1 or 2 days contain aggregates. From the appearance of such samples on acrylamide gel it can be estimated that at most 10% of the material is somewhat retarded compared with the major band. An approximate s value of 20 S would characterize this component as a dimer of the major species present. An additional aggregate of higher order can be seen on acrylamide but not in the ultracentrifuge, and represents at most 1% of the sample. Using the relation $M_{w,obsd} = \Sigma M_i c_i / \Sigma c_i$ with the assumption that 10% of the material analyzed is a dimer of the major species and neglecting the presence of higher aggregates, one arrives at a value of 326,000 for the major species. Since the Yphantis method specifically allows estimation of the lowest molecular weight species in the presence of higher molecular weight material, whereas the low-speed sedimentation to equilibrium gives a weight-average molecular weight of all species present, the different values obtained by these methods are to be expected. The apparent molecular weight of the protein dissolved in 5 M Gd·HCl, pH 7.1, was 62,000 using high-speed sedimentation to equilibrium at 30,000 rpm at a protein concentration of 0.6 mg/ml. A preparation which had been reduced and alkylated with iodoacetamide in 5 M Gd·HCl never cleared the meniscus even at 40,000 rpm. This observation remains unexplained. Since diffusion studies indicated that the tendency of the protein to form aggregates was increased by thiol compounds in the presence of high salt concentration, Gd·HCl was not supplemented by thiol compounds. The value of 1.12 used for the density of the 5 M Gd·HCl solution was taken from Kawahara and Tanford (1966).

Other Attempts in the Elucidation of the Structure of CF_1. Attempts to probe into the subunit structure of the protein by chemical methods met largely with failure. The method of Stark (1964) for the quantitative determination of N-terminal amino acids failed to reveal any free N termini suggesting that the N termini may be blocked. Since recoveries of blocked N termini are known to be low and variable, their determination was not pursued. An alternative way to establish the number of polypeptide chains is to determine the number of arginine- and lysine-containing peptides after tryptic digestion. This approach was also unsatisfactory because tryptic digestion of the protein invariably gave up to 40% "core" material. The only indication that the protein might consist of identical repeating units came from cleavage with cyanogen bromide by the method of Gross and Witkop (1962). Cyanogen bromide very selectively cleaves polypeptides at methionine residues, liberating their α-carboxyl group from the peptide linkage. The reaction was carried out in the solvent system used by Steers *et al.* (1965) and analysis of the products on polyacrylamide gel revealed eight clearly resolved zones; since amino acid analysis showed the presence of seven methionine residues per cysteic acid residue, a repeating unit having a molecular weight of 28,000 (calculated per cysteic acid) is suggested. The apparent molecular weight of a species forming upon dissolution of the protein in 5 M Gd·HCl would thus represent a subunit of approximately twice the minimal molecular weight.

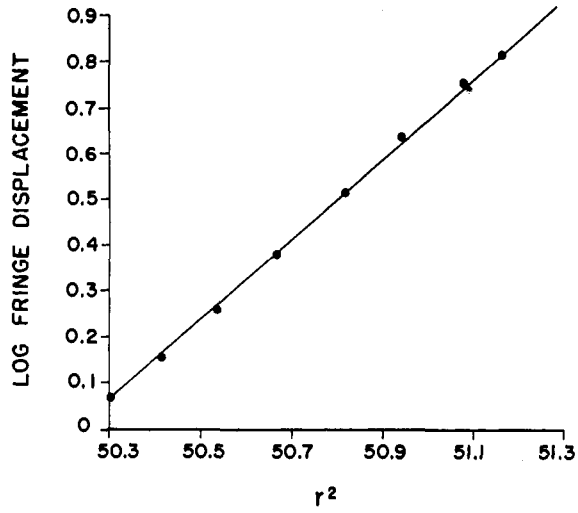

FIGURE 4: Dependence of log c on r^2 during low-speed sedimentation to equilibrium. The rotor speed was 4400 rpm. The protein concentration was 0.6 mg/ml. The temperature was 14°. Equilibrium was reached after 31 hr.

Comparison of Native Coupling Factor and Heat-Activated ATPase by Circular Dichroism. From the shape of the circular dichroism in the peptide absorption region investigated (240–190 mμ), it can be concluded that most, if not all, of the ordered secondary structure in CF_1 is α helical. On the basis of this assumption (no β structure) a helix content of 20 ± 3% was calculated taking the value of helical α-polyglutamic acid as 100%. The uncertainty in the helix content arose from changes in the spectrum of a solution of CF_1 which occurred upon standing at room temperature for a few hours. Within the limits of the above error, there was no difference in the amount of α helix between activated and latent ATPase. These data do not exclude changes in tertiary or quarternary structure upon activation.

Since the optical activity properties of the aromatic side chains are sensitive to environmental changes, the circular dichroism in the aromatic (and cystine) absorption region (300–240 mμ) was investigated. Here again, there was no significant difference between activated and latent ATPase. $[\theta]$ at 270 mμ was −33 deg cm²/dmole for both forms; experimental uncertainty in this region of the spectrum was considerable because the signal to noise ratio was approximately 1.5. These data represent weak negative evidence against extensive tertiary rearrangement upon activation, especially since CF_1 has a low content of aromatic residues. However, in conjunction with electrophoretic and ultracentrifugal studies presented, the conclusion seems warranted that the changes induced by controlled heat treatment are localized and without profound effect on the tertiary structure of the molecule.

Acknowledgments

I wish to express my deep appreciation to Dr. Efraim Racker for his encouragement and continued interest during the course of this work and my sincere thanks to Dr. Stuart Edelstein for helpful discussions relating to experiments in the ultracentrifuge. The competent assistance of Mr. James Finucane in the large-scale preparation of the enzyme is gratefully acknowledged.

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