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HYDROGEN EXCHANGE INTO SOLUBLE SPINACH CHLOROPLAST COUPLING FACTOR DURING HEAT ACTIVATION OF ITS ATPase

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Exchange of 500–600 atoms of ^3H per mol of solubilized spinach chloroplast coupling factor (CF_1) occurs when the enzyme is incubated for 4 min in ^3H_2O at 63°C. These ^3H atoms are bound in parts of the protein where exchange is hindered by the three-dimensional structure at 25°C. Back-exchange at 25°C shows complex kinetics, with at least two kinetic components having half-times of 1.4 and 40 h, respectively. Back-exchange from the denatured enzyme is extremely rapid with an apparent half-time of the order of 20–30 s. The time courses for exchange and ATPase activation are very similar at 63°C, and reasonably close at 25°C. Both reactions have an optimum temperature of 60°C when measured after 4 min. Activation of ATPase requires a strong reducing agent to be present, but this is not needed for hydrogen exchange. It is suggested that an open conformation of CF_1 induced by heat may be a required intermediate for the rapid activation of ATPase, being a sporadic and rare occurrence at 25°C but also a required step in ATPase activation. This open conformation could be related to that induced in bound CF_1 by thylakoid membrane energization.

Introduction

Photophosphorylation in chloroplast thylakoid membranes is catalyzed by an ATPase complex, one part of which (CF_1) is easily detached from the membranes and has been purified [1]. Soluble CF_1 has latent ATPase activity, which may be activated by digestion with trypsin or by heating [2]. When on the membrane, rapid activation of ATPase occurs in the presence of strong reducing agents and membrane energization [3–6]; and the latter has been shown both by accessibility of inhibitors [7–14] and hydrogen exchange studies [15,16] to cause significant conformational change in CF_1. With the soluble enzyme, accessibility to inhibitors was found to occur only during the heat-activation process [17,18]. The current work was an attempt to see if hydrogen-exchange techniques would also reveal significant conformational changes in soluble CF_1 during heat activation.

Protons attached to N, O and S atoms of molecules dissolved in water are, in general, able to exchange with mobile protons in the solvent [19]. It is generally assumed that the marked contrast between exchange kinetics of native (folded) proteins and random polypeptides is primarily due to solvent inaccessibility of many hydrogen peptide or other groups in the three-dimensional structure [19,20]. Thus, measurements of the kinetics of hydrogenexchange can provide a probe for identifying and quantitating conformational changes of proteins or other macromolecules.

Abbreviations: SDS, sodium dodecyl sulfate; sodium Tricinate, sodium salt of *N*-tris(hydroxymethyl)methylgycine; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.

Materials and Methods

Materials

Sephadex G-50 fine was purchased from Sigma or Pharmacia; dithioerythritol, ATP, *N*-ethylmaleimide, dithionitropyridine and iodosobenzoate were all purchased from Sigma. ^3H_2O was purchased from New England Nuclear Corp.

Chloroplasts were prepared from market spinach; CF_1 was solubilized by treatment of the membranes at low ionic strength and purified by ammonium sulfate precipitation and either DEAE-Sephadex chromatography [21,22] or sucrose density gradient centrifugation [23]. Similar results were obtained with both types of preparation.

The ATPase of CF_1 was activated by incubation at 63°C for 4 min, in the presence of ^3H_2O at 1.0 mCi/ml when hydrogen exchange was to be measured. Incorporation of ^3H was tested for by retention of the counts when the protein was passed through one or two small gel filtration columns, using the rapid centrifugation-filtration technique of Penefsky [24]. Tests of the procedure showed that all but 1 part in 10^8 of the supplied radioactive free water can be retained in a carefully prepared column, while the protein appears quantitatively in the eluate. With less careful techniques as much as 1 part in 10^5 of the original free water may appear in the eluate, but then the use of two successive columns permits an entirely effective separation.

The reaction mixture for heat activation included 50 mM sodium Tricinate (pH 8.0), 2 mM EDTA, 40 mM ATP, 5 mM dithioerythritol and CF_1 at 0.5–2.0 mg/ml [20]. After heating, the reaction mixture (100 or 200 μl) was applied to a 1.0 or 2.0 ml volume column of Sephadex G-50 fine contained in a disposable syringe. The Sephadex had previously been equilibrated with 50 mM sodium Tricinate (pH 8.0), 2 mM EDTA and 1 mM ATP, centrifuged, and stored at 0°C. The syringe with reaction mixture applied was centrifuged again, with protein appearing in the eluate. After 10 min at room temperature (to permit back-exchange of superficial hydrogen atoms), the eluate was passed through a second Sephadex column with centrifuging. In an alternative procedure the labeled CF_1 was passed through one static column (0.7 × 12 cm) of Sephadex G-50 fine. Similar results were obtained with both methods. Controls included additions of SDS to the CF_1 either before the first, or between the first and second columns; and zero-time controls in which the ^3H-containing reaction mixture was added to the first column, followed by CF_1 with immediate centrifuging. The final eluates were assayed for protein [25], ATPase [2] and radioactivity.

ATPase of the activated enzyme was determined in a 1.0 ml reaction mixture containing 50 mM Tris-HCl (pH 8.6), 5 mM $CaCl_2$ and 2–6 μg CF_1. The reaction proceeded for 5 min at 37°C, and then was stopped by addition of 1 ml of 8% (w/v) trichloroacetic acid followed by 1 ml of the Taussky and Shorr [26] reagents for measuring released P_i. Alternatively, the reaction was stopped and P_i measured by addition of the reagent described by Lebel et al. [27]. Protein was measured with the Coomassie blue G dye-binding method [25] using as standards either bovine serum albumin (concentration determined spectrophotometrically with 10 mg/ml assumed to have A_{279} = 6.67), or standard CF_1 solution the concentration of which was determined spectrophotometrically [1]. CF_1 was found to have approx. 67% as much dye-binding ability per mg protein as bovine serum albumin did, when protein was measured by the method of Lowry et al. [28], so dye-binding protein values were multiplied by 1.5.

Most radioactivity measurements were made in a Beckman L-233 liquid scintillation counter using 5 ml of scintillation fluid (5 g PPO and 100 g naphthalene in 1 l of 1,4-dioxane), with efficiencies of 25–30% determined by an external standard. Other measurements were made with a Triton/toluene/PPO/POPOP mixture in a Packard model 3 255 liquid scintillation spectrometer at about 40% efficiency. The number of ^3H atoms per mol CF_1 was calculated from the known specific activity of 1 mCi/55 mmol H_2O (=40 dpm/nmol; thus, 1 dpm represents 0.025 nmol) and a molecular weight of CF_1 of 325 000 (so 1 μg = 0.00308 nmol). Thus, 1 dpm/μg protein represented 8.12 nmol ^3H_2O or 16.25 natom ^3H/nmol CF_1.

Results

Some incorporation of ^3H into CF_1 in a form not lost on gel filtration occurred at all temperatures (Table I). However, given a 4 min incubation period, 6-times as much occurred at 63°C as at 20°C, and 10-

TABLE I

INCORPORATION OF ^3H INTO CF_1 DURING ACTIVATION OF ITS ATPase BY HEATING

Incubations were for 4 min as described in Materials and Methods and in the text. Room temperature was 22°C. When present, SDS was added between the two column purification steps, which was also the point at which reheating of the CF_1 protein was done. In other experiments zero-time controls gave the same level of ^3H incorporation as was found for the SDS-treated samples. Data of $^3H/CF_1$ represent atoms/mol protein; ATPase is shown in μmol P_i released/min per mg protein.

Conditions	$^3H/CF_1$	ATPase
Complete	517	18.8
Incubation at room temperature	74	2.5
Incubation at 0°C	56	2.5
SDS added	10	–
Dithioerythritol	527	11.6
2nd heating in ^1H_2O	113	19.9

times as much as at 0°C (Table I). Some ATPase activity was present without heating in these preparations of CF_1, but most activity was obtained after incubation at 63°C. The incorporated ^3H was lost very rapidly when SDS was added prior to a column step, indicating the need for native protein structure to maintain the incorporated ^3H. Denaturation by urea caused similar rapid loss of ^3H, and the zero-time controls had virtually no incorporation. Dithioerythritol was required for maximum activation of the ATPase but its omission did not change the amount of ^3H incorporated into CF_1. Reversibility of the incorporation was shown by putting the labeled CF_1 into a second heating step with only ^1H_2O, once the free ^3H_2O had been removed by gel filtration (last row, Table I).

Maximal amounts of ^3H incorporation were only obtained if the reaction mixture was applied to a precooled (0°C) Sephadex column; when applied to a column at room temperature the observed incorporation dropped by 20%. Probably some back-exchange occurs on the column if the heated CF_1 is diluted, but comes to temporary equilibrium at a temperature above that of the ambient atmosphere. Incubation and centrifugation had to be performed in a hood for considerations of radiation safety, hence the need to precool the column rather than work in a cold room.

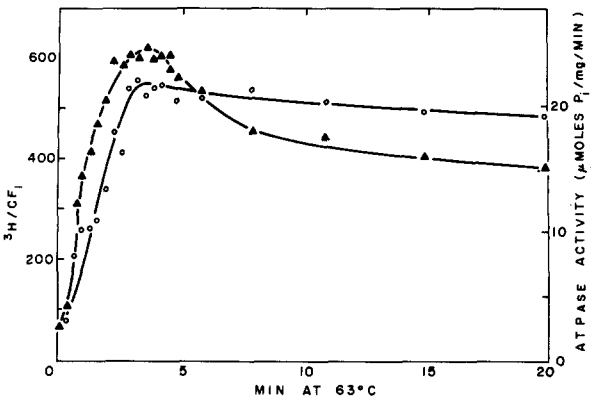

Fig. 1. Time course of incorporation of ^3H into CF_1 during heat activation. A reaction mixture of 1.5 ml volume was heated at 63°C, with ^3H_2O at 1.0 mCi/ml. At the times shown, aliquots of 0.10 ml were centrifuged through 1 ml Sephadex G-50 columns preequilibrated with the standard buffer at 0°C. After a 5 min wait, the eluate from each column was centrifuged through a second Sephadex column at room temperature. Protein, ATPase and radioactivity levels were determined on the final eluates. ATPase units are μmol P_i /mg protein per min. In two control aliquots taken from the reaction at 2 and 10 min, SDS was added to a concentration of 1% to the eluate from the first column. After passing through a second column, these retained 16 and 28 $^3H/CF_1$, respectively. (▲) ATPase activity, (○) $^3H/CF_1$.

The time course for incorporation of ^3H at 63°C showed a maximum achieved by 4 min, at which point the CF_1 had about 500–600 ^3H atoms per mol (Fig. 1). The incorporation of ^3H, and activation of ATPase, showed very similar kinetics, although enzymatic activity decayed with increasing time at 63°C while the ^3H incorporation essentially did not.

At 25°C, by contrast, both ^3H incorporation and enzyme activation were much slower (Fig. 2). The number of ^3H atoms reached the same level as at 63°C (about 500–600/mol CF_1) but took 3 h to reach that level instead of 4 min. In this experiment the activation of ATPase seemed to be a little faster, with the optimum reached after 2 h of incubation. Also, as in previous work [29], activation of the soluble ATPase at room temperature is completely dependent on adding a strong reducing agent such as dithioerythritol. Incorporation of ^3H has no such requirement.

In other experiments (not shown), ATPase previously activated by heating was incubated with ^3H_2O

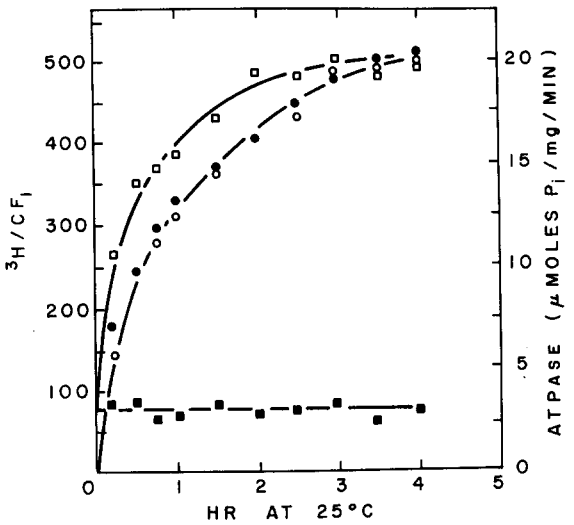

Fig. 2. Time course for incorporation of ^3H into CF_1 at room temperature. A standard reaction mixture was made up containing ^3H_2O at 0.5 mCi/ml, 2 mg/ml CF_1, 40 mM ATP, 50 mM sodium Tricinate at pH 8.0, 3 mM NaN_3 to control bacterial growth, with (○, □) or without (●, ■) 10 mM dithioerythritol (DTE) as indicated at the times shown. Aliquots were passed through 0.7 × 12 cm Sephadex columns with a 10 min wait in the middle of the column. Protein and radioactivity were measured in the column eluents. (□, ■) ATPase activity, (○, ●) $^3H/CF_1$.

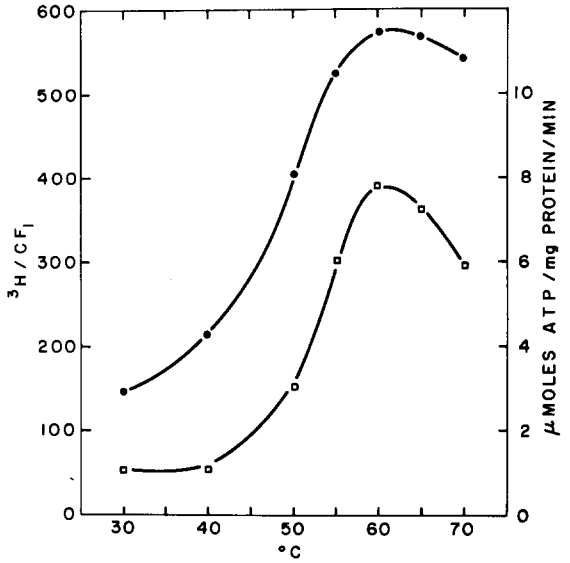

Fig. 3. Temperature dependence of ^3H incorporation. CF_1 at 2 mg/ml was incubated in the standard buffer and ^3H_2O (1.0 mCi/ml) for 4 min at the temperatures shown, in separate tubes containing 0.08 ml reaction mixture. The reaction medium was equilibrated at the desired temperature for 2 min prior to addition of CF_1. After the incubation, the samples were centrifuged through two Sephadex columns, and measurements of protein, ATPase and radioactivity were performed as described in Materials and Methods. (□) ATPase activity, (●) $^3H/CF_1$.

at room temperature as in Fig. 2. However, it did not show any more rapid incorporation of ^3H than did the latent ATPase of Fig. 2; thus, the rapid incorporation occurs only during the heating step, not before or after.

The temperature dependence for incorporation of ^3H during a 4 min incubation is shown in Fig. 3. Some incorporation occurred at all temperatures, but the optimum rate did not appear until 60°C. The ATPase was not stimulated by brief incubation at 30 or 40°C, but ^3H incorporation was. However, the optimum temperature for both reactions was found to be at 60°C. As might be expected, the enzyme activity was damaged by higher temperatures, while the ^3H incorporation was fairly stable at 65 and 70°C.

The back-exchange of ^3H from labeled CF_1 with ^1H_2O at room temperature was studied by varying the interval between the first and second centrifugation-filtration column steps (Fig. 4). After the first column step the ^3H content of the solution had been reduced by at least 10^{-5}, so the interval between the two column steps was a time during which back-exchange could occur. The curve for ^3H remaining after the second column step was complex, but seems to resolve into three apparent first-order components when plotted on a semilogarithmic basis (Fig. 4). The fastest showed a half-time of about 3 min, with approx. 1 000 H atoms/CF_1 falling into this category. This rapidly decaying incorporation of ^3H was not observed in the standard experimental protocol, in which a 10 min wait between the first and second column steps had been routine procedure. The intermediate speed component showed a half-time of 1.4 h, and the slowest one of 40 h; with 400 and 120 H atoms/CF_1 in these categories, respectively.

Back-exchange from denatured CF_1 was extremely rapid. For these experiments labeled CF_1 was passed through one Sephadex column and large amounts pooled. One aliquot of 0.3 ml was centrifuged through a second Sephadex column; the eluted protein served as a control. A second aliquot was applied to a Sephadex column containing 0.1% SDS, and

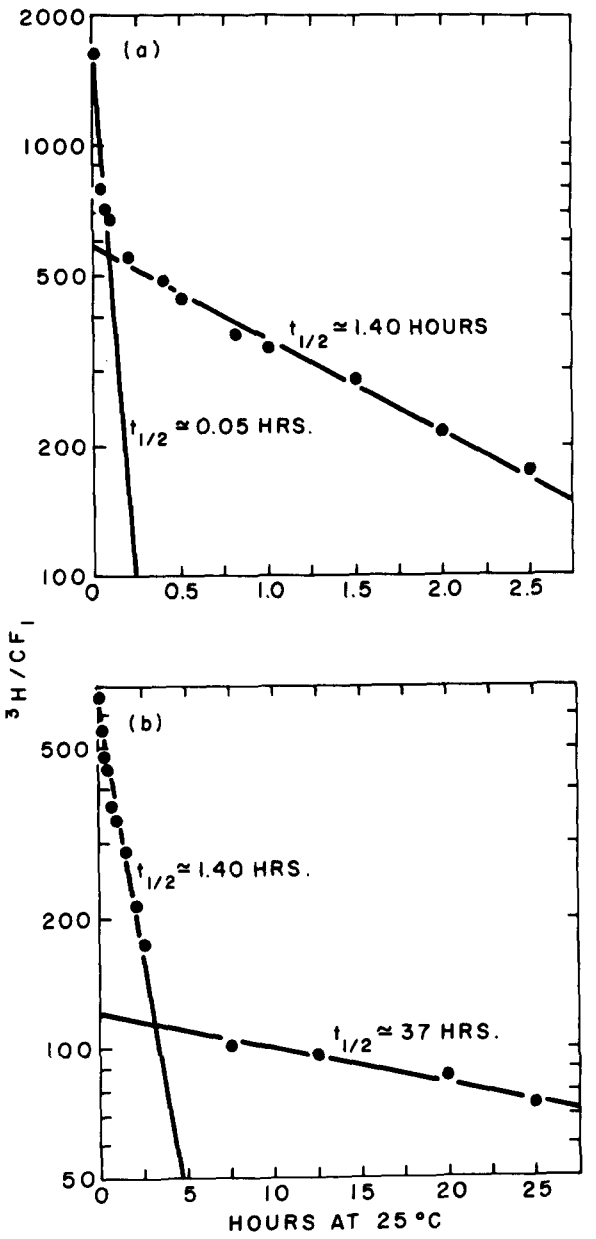

Fig. 4. Back-exchange in ^1H_2O at 25 C. CF_1 was labeled by incubation for 4 min in 1 mCi/ml of ^3H_2O as described in Materials Methods. The reaction mixture was cooled to 0°C for 30 s then filtered through a Sephadex column equilibrated with standard buffer containing 3 mM NaN_3 to control bacterial growth. The eluate was kept at 25°C in a water bath. At the times shown aliquots were removed and passed through a second Sephadex column and centrifuged. Protein and radioactivity measurements were made on the eluates from the second columns.

centrifuged immediately. This served as the 'zero-time' sample (with respect to time of exposure to SDS). Other samples were incubated in 1% SDS for 30 and 60 s, before centrifuging through a Sephadex column containing 0.1% SDS. The 0, 30 and 60 s samples had 34, 17.5 and 6.5% as much ^3H, respectively, as did the nondenatured control. Thus, an approximate half-time of 20–30 s can be inferred for the back-exchange from denatured CF_1.

The effects of some SH-binding inhibitors on ^3H incorporation were examined (Table II). These were applied during the preliminary heating step at 63°C [17]; then the modified protein was exposed to ^3H_2O in a second heating step. The ATPase activity had been inhibited to 60% by all three reagents after the first heating step, but some activity seemed to be restored following the second exposure to 63°C. Dithionithropyridine (0.25 mM) and *o*-iodosobenzoate (10 mM) inhibited ^3H incorporation 15–20%, but

TABLE II

EFFECT OF PREVIOUS MODIFICATION OF THE ENZYME WITH THIOL GROUP REAGENTS ON ATPase ACTIVATION AND ^3H EXCHANGE CAUSED BY A SECOND HEATING STEP

CF_1 samples were heated in buffer lacking ^3H_2O but containing inhibitors as shown. After passage through Sephadex to remove the inhibitors, ATP and ^3H_2O were added and all samples were heated a second time at 63°C. ATPase rates are in μmol ATP split/mg protein per min. *o*-Iodosobenzoate (IBZ) and *N*-ethylmaleimide (MalNEt) were used at 10 mM, dithionitropyridine (DTNP) at 0.25 mM. The addition of dimethyl sulfoxide (DMSO) to 10% served as a control for DTNP, which was dissolved in DMSO as a 10-fold concentrated stock solution. Three additional experiments (not shown) gave almost identical percent inhibition results (16 and 15% for DTNP, 21% for IBZ, 0% for MalNEt inhibition of hydrogen exchange). Exchange data are given as atoms ^3H/mol CF.

Addition	ATPase		Exchange	
	Rate	Inhibition (%)	$^3H/CF_1$	Inhibition (%)
–	12.5	–	550	–
DMSO	12.3	2	525	5
DTNP	8.1	34	414	21
IBZ	8.6	31	449	18
MalNEt	7.0	56	551	0

N-ethylmaleimide had no effect on the exchange reaction (Table II).

Attempts were made to see if the incorporated ^3H represented tightly bound water rather than hydrogen atoms bound covalently. Aliquots of CF_1 were frozen, lyophilized, and then reconstituted in buffer. Assays of ATPase, protein and ^3H retained after lyophilizing indicated variable loss of enzyme activity, and a loss of ^3H corresponding roughly to the loss of enzyme activity. Presumably, back-exchange occurred very rapidly as the protein was denatured during the freezing process, or even in the frozen condition, and this radioactivity was lost with the removal of free water. In three experiments in which no loss of enzyme activity occurred (helped in part by the addition of 10 mM raffinose to the freezing mixture) 88% or more of the radioactivity remained with the reconstituted protein. Thus, if there is some bound water present in CF_1 purified by gel filtration, it must account for only a small part of the observed radioactivity.

Discussion

The method used here for ^3H-exchange studies is similar to that used by Englander [30]. However, separation of the labeled protein from free water occurs in about 15 s with the centrifugation-filtration procedure [24] compared to the 2 min estimated for a static Sephadex column [30]. This more rapid resolution time permits the study of more rapidly exchangeable hydrogen atoms, having half-lives of the order of 1 min or less.

The results presented here give quantitative evidence that CF_1 has a considerably more open conformation at 60°C than at room temperature, and the exchange of about 500–600 H atoms per mol occurs rapidly from the open conformation but is restricted by a closed conformation at room temperature. A more open conformation exposing at least one sensitive -SH group was previously inferred from the fact that dithionitrophyridine and *o*-iodosobenzoic acid did not modify the protein at the sensitive -SH group at room temperature, but did so at 63°C [17,18]. These, and previous results, permit the definition of two possible working hypotheses for the events occurring during activation of the latent ATPase of soluble CF_1: (1) Latent and active ATPases differ both in conformation, and in the distribution of -SS- groups [18]. (2) The transition from the conformation of the latent to that of the active ATPase can occur slowly at room temperature (and is stabilized only if disulfide interchange, initiated by the added dithioerythritol, occurs) but is much more rapid from the open, intermediate conformation found at 63°C. (3) In this model, the active ATPase is at a lower energy level than the latent conformation.

In the alternative hypothesis the latent and active ATPase may have essentially the same conformation, but the disulfide interchange events could require an intermediate, open conformation. This permissive conformation may occur rarely and at random at room temperature, but would be a prominent feature of the much more open enzyme found at 63°C.

In either case, a more open conformation of the protein is likely to be a necessary intermediate during ATPase activation. Correlations between ATPase activation and hydrogen atom exchangeability (Figs. 1–3) can be looked at from this point of view. There is a very reasonable similarity between the time course for activation of ATPase at 63°C, and that for hydrogen exchange (Fig. 1). Enzyme activity falls off with time; however, slight denaturation would be expected to affect this function more sensitively than that of the nonenzymatic hydrogen exchange reaction. The correlations are fairly close but not perfect with respect to exchange and ATPase at room temperature (Fig. 2), and for the temperature curve (Fig. 3). However, the exposure of internal groups to permit hydrogen exchange is a very general property of the protein, and presumably the postulated intermediate conformation of CF_1 needed for disulfide interchange (or for the jump to the active conformation) is restricted to a specific conformation out of the numerous ones able to exchange H atoms with solvent water rapidly. This could easily account for the increase in hydrogen exchange during 4 min at 30 or 40°C compared to 25°C, with no perceptible enzyme activation yet. We consider it more significant that they both reach an optimum extent at the same temperature, 60°C.

Examination of the kinetics of back-exchange of the labeled protein at 25°C (Fig. 4) shows the existence of at least three kinetic 'classes' of hydrogen exchange. All three of them are slower than the exchange out of labeled protein denatured by SDS

(where the half-time is of the order of 30 s), hence with the native enzyme, the protein structure must restrict access of the exchanging groups to solvent water to different degrees. These results, and the interpretation, are similar to those reported by Englander [30], for instance, with labeled RNAase. Hvidt and Nielsen [19] proposed a model in which exchange occurs only from exposed groups, and thus different kinetic classes would correspond to different degrees or mechanisms of unfolding needed for exposure. However, Woodward et al. [20,31] suggested a more complex model in which the exchange of a peptide amide proton occurs either directly from the folded protein, or via unfolding to permit exposure. Most peptide amide protons in a folded protein could exchange with the solvent without unfolding, during structural fluctuations between folded conformations. This sort of fluctuation would have a low activation energy, and correspond to the concept denoted a 'breathing' mechanism [20] with simultaneous breaking and reforming of several hydrogen bonds. Of our kinetic classes (Fig. 4), the two slowest ones, representing those examined in most of the experiments of this paper, probably relate to 'masked' or 'buried' bonded hydrogen atoms in the three-dimensional structure of the native protein.

The most abundant source of exchangeable H atoms in a protein is the group bound to the N atom of the peptide bonds. Each CF_1 contains about 2 870 such peptide amide hydrogens (calculated from data in Ref. 23 and subtracting the values for proline). Hence, the exchange of about 550 from relatively hidden locations amounts to 20% of the total available. It may be relevant that Farron calculated 20% of the structure of CF_1 (either native or heat activated) to be in α-helices [1].

When bound to thylakoid membranes, the ATPase of CF_1 is latent until activated by membrane energization and a strong reducing agent [3–6]. The same membrane energization has been shown in a number of ways to cause a fairly large conformational change in CF_1 [7–16,32]. It would be interesting to know how closely the open configuration of CF_1 on energized membranes resembles that of the soluble enzyme at 63°C. By the criterion of the number of exchangeable H atoms exposed to the medium only in the open configuration, the open conformation on the membranes is likely to be more limited. ^{3}H-incorporation studies [16] indicated up to 100 ^{3}H atoms per mol of CF_1 exchanging into internal parts of the molecule on energized membranes, compared to the 500 or more with soluble CF_1. However, in the previous studies, the methods used required 2 h or more between illuminating the chloroplasts and measuring radioactivity on purified CF_1. During this period, it is certain that some back-exchange was occurring so the number of 100 per mol is only a minimal estimate. It may be rewarding to try to repeat the earlier experiments with a more rapid or more effective technique, to see if the amount of internal H atom exposure with membrane-bound CF_1 might be closer to that with heated soluble CF_1 than we are now able to say. If the numbers do turn out to be closer than now thought, then the heated-induced conformational changes of CF_1 in solution could turn out to be a useful, somewhat simpler model system to help understand the nature of the reversible changes induced in bound CF_1 by membrane energization.

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