INTERACTION OF ADENINE NUCLEOTIDES WITH THE COUPLING FACTOR OF SPINACH CHLOROPLASTS

A hydrogen–deuterium exchange study

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1. Introduction

Hydrogen exchange is a sensitive method for detecting subtle structural changes that may occur when a ligand binds to a protein [1–5]. In the present paper we compare the interaction of coupling factor 1 with different adenine nucleotides (AMP, ADP, ϵATP, ATP) by means of hydrogendeuterium exchange measurements to get information about the compactness of the liganded and unliganded forms. Coupling factor 1 is a five-subunit enzymatic protein [6] which catalyses the formation of ATP from ADP and inorganic phosphate during photosynthesis. Circular dichroism or fluorescence measurements [7,8] indicate that the interaction of CF_1 is equally strong with ATP, ϵATP, or ADP but absent with AMP and that it corresponds to a nucleotide binding on three sites of CF_1. The present results also show that all of the nucleotides studied, except AMP, increase the conformational stability of the enzyme.

2. Materials and methods

2.1. *Extraction and purification of CF_1*

CF_1 was solubilized from spinach chloroplasts by 10^{-3} M EDTA [7] and purified according to Lien and Racker [9]. The purity of CF_1 was checked by polyacrylamide gel electrophoresis. Protein concentrations were determined by ultraviolet absorption [8,10].

Abbreviations: CF_1, coupling factor 1; ϵATP, 1,N^6-ethenoadenosine triphosphate; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism

2.2. *Biochemical activity of CF_1*

CF_1 was heat activated [11] and its ATPase activity measured: an aliquot containing 2×10^{-5} g of CF_1 was transferred to the thermostated cuvette (37°C) of a pH-stat in a medium containing approximately 3×10^{-2} M ATP and 3×10^{-2} M $CaCl_2$. The pH value was continuously adjusted to 8.0 by the addition of 10^{-2} M NaOH.

2.3. *Circular dichroism spectra*

CD Measurements were performed in a Roussel Jouan CD II dichrograph, with a 0.1 mm optical pathlength.

2.4. *^1H–^2H exchange*

Prior to exchange experiments, nucleotide–CF_1 complexes were formed by mixing CF_1 (8×10^{-5} M) and a nucleotide (2×10^{-3} M) for 17 h at room temperature, in 2×10^{-2} M Tris buffer, 2×10^{-3} M $MgCl_2$, with pH values ranging from 7.24–8.14. Then, samples were lyophilized. The ^1H–^2H exchange reaction was investigated by infrared spectroscopy [12,13] following the previously described experimental procedure [14,15]. Infrared spectra were scanned at 19.5°C between 1800 cm^{-1} and 1300 cm^{-1} on a Perkin Elmer 521 double beam spectrophotometer equipped with matched, thermostated cells.

The fraction X of unexchanged peptide hydrogen/mol CF_1 was estimated as

$$X = \frac{1}{w} \left(A_{\text{amide II}}/A_{\text{amide I}}\right) \quad (1)$$

$A_{\text{amide II}}$ is the absorbance at the maximum of the

amide II band (due to CO–NH groups), $A_{\text{amide I}}$ is the absorbance at the maximum of the amide I band (due to C=O group of the peptide bonds) and w is the ratio $A_{\text{amide II}}/A_{\text{amide I}}$ for the undeuterated protein; w was taken as 0.45 [12–15].

The baseline of the amide II was taken as the absorption of a solution of the completely deuterated protein (CF_1 incubated at 45°C for 24 h in buffered ^2H_2O, at the appropriate pH); similarly completely deuterated complexes were obtained after 30 h in the same buffer at 50°C. $A_{\text{amide II}}/A_{\text{amide I}}$ ratios obtained for AMP–, ADP–, ϵATP–, ATP–CF_1 complexes and for free CF_1 were 0.195, 0.195, 0.192, 0.197 and 0.187, respectively.

The reported pH values were measured on ^2H_2O solutions with standardization against buffer prepared with ^1H_2O.

3. Results and discussion

Infrared spectra of CF_1 in ^2H_2O (2×10^{-2} M Tris, pH 8.14) at two stages of exchange (17 min and 23 h 52 min) are displayed in fig.1. The amide I band has its maximum at 1633 cm^{-1} and a shoulder at 1650 cm^{-1} which are assignable to the presence of a significant amount of β-structure and a small portion of α-helix, respectively [16,17]. The carboxylate band

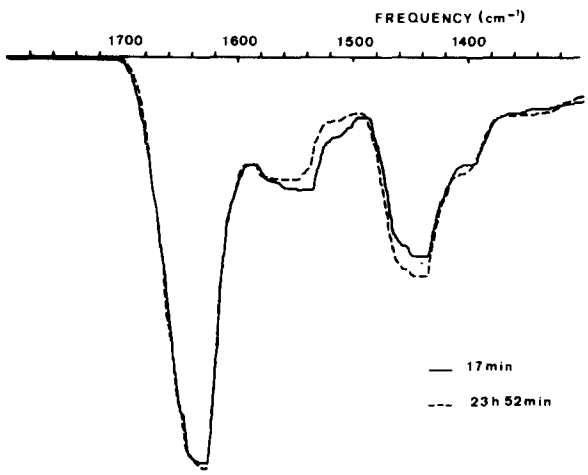

Fig.1. Infrared spectra of CF_1 (1.33×10^{-4} M) in ^2H_2O (2×10^{-2} M Tris, pH 8.14) at 19.5°C after 17 min (——) and 23 h 52 min (- - -) exchange times.

at 1575 cm^{-1} [18] overlaps the amide II band at 1540 cm^{-1}, the absorbance of which decreases on deuteration, while a new band appears at 1443 cm^{-1} due to both $-N^2H$ and $^1HO^2H$ absorption. The observed frequencies (with an accuracy of ± 1 cm^{-1}) and shape of the amide I bands of nucleotide–CF_1 complexes are identical to those of native CF_1, suggesting that the secondary structures are similar.

Parallel measurements with the most concentrated CF_1 solutions (3.9×10^{-6} M) for which CD spectra can be measured suggest a non-negligible proportion of α-helix. For a better comparison of CD and infrared observations, infrared spectra of CF_1 were recorded as a function of concentration, revealing a progressive shift of the amide I peak from 1633 cm^{-1} (15.4×10^{-5} M CF_1) to 1640 cm^{-1} (3.1×10^{-5} M), then to 1648 cm^{-1} with a shoulder at 1638 cm^{-1} (1.54×10^{-5} M). According to the assignments of the amide I frequencies [16,17], these observations furnish strong evidence of a transition of CF_1 from a prevalent β-conformation to a juxtaposition of α-helical, β- and unordered structures, as the concentration is decreased. With histones, a similar conformational change as a function of concentration was recently described [19].

For a more quantitative comparison of infrared and CD spectra, experimental spectra were fitted as sums of published reference spectra [20–22] using a general least squares program developed for a PDP 12 computer; infrared data analysis confirms a change in conformation when the concentration decreases from 15.4×10^{-5} M ($\alpha = 7, \beta = 91, \gamma = 2$) to 1.54×10^{-5} M ($\alpha = 41, \beta = 17, \gamma = 42$) where α, β, and γ are the respective percentages (within ± 20%) of α-helix, β-conformation, and random structure. From CD spectra, at 3.9×10^{-6} M, the following percentages were calculated: $\alpha = 21, \beta = 4, \gamma = 75$ (within ± 10%). These agree with the infrared results at the lowest concentration and with previous CD results [10].

Kinetic exchange data were analysed in terms of the mechanistic scheme proposed by Hvidt and Nielsen [23], according to which the fraction X of unexchanged peptide hydrogen/mol protein at time t is given by

$$X = n^{-1} \sum_{i=1}^{n} \exp(-\rho_i k_0 t) \qquad (2)$$

where n is the total number peptide groups, ρ_i is the

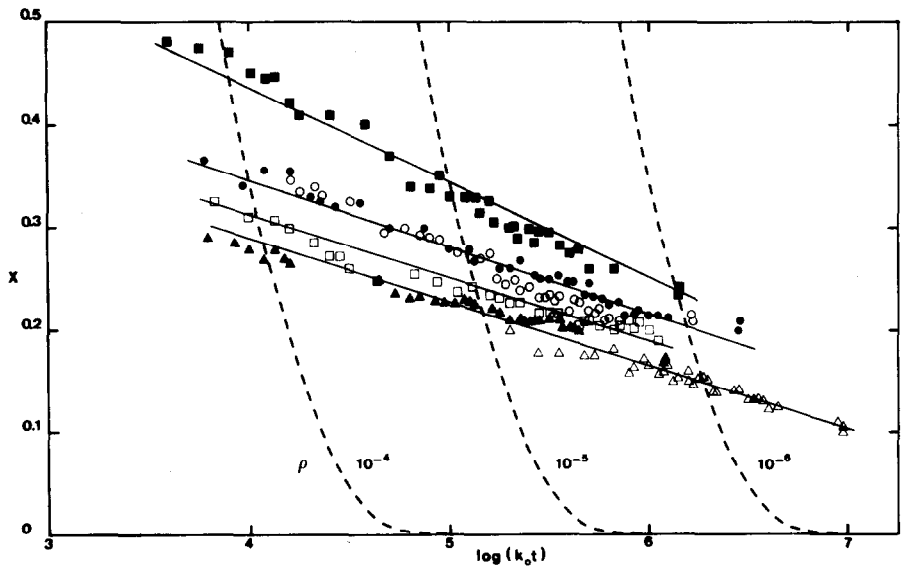

Fig.2. Comparison of $^1H-^2H$ exchange data at 19.5°C for CF_1 ((▲ ▲) pH 7.24, (△ △) pH 8.14) and the following nucleotide–CF_1 complexes: AMP–CF_1 ((□ □) pH 7.56), ADP–CF_1 ((● ●) pH 7.62), ϵATP–CF_1 ((○ ○) pH 7.40) and ATP–CF_1 ((■ ■) pH 7.33); X is the fraction of unexchanged peptide hydrogens at time t, k_0 is the exchange rate constant of solvent exposed peptide groups (eq. 3). Broken curves are calculated for hypothetical polypeptides with ρ values indicated in the figure (ρ is the probability of solvent exposure of the peptide hydrogens).

probability of finding the peptide i exposed to solvent and k_0 is the exchange rate constant of solvent-exposed peptide groups [23]

$$k_0 = 50\,(10^{-pH} + 10^{pH-6})10^{0.05\,(\theta-20)}\ \text{min}^{-1} \quad (3)$$

θ is the temperature in centigrade.

Figure 2 shows the exchange curves for CF_1 in the presence of adenine nucleotides, as plots of X versus $\log(k_0 t)$; this representation was chosen because it allows the comparison of data obtained at different pH values, i.e., for different values of k_0 [24].

Kinetics of CF_1 were followed at pH 8.14 and pH 7.24, those of nucleotide–CF_1 complexes at pH 7.56, pH 7.62, pH 7.40 and pH 7.33 for AMP–, ADP–, ϵATP–, and ATP–CF_1 complexes, respectively. In the same figure is represented a part of the $X = \exp(\rho k_0 t)$ curves corresponding to hypothetical polypeptides for which ρ has the following values: 10^{-4}, 10^{-5}, 10^{-6} [25]. The smaller the ρ value, the more deeply a peptide proton is buried in the hydrophobic internal regions of the molecule. If a NH group is freely accessible to the solvent, $\rho = 1$. Table 1 gives a summary of the distribution of ρ values in CF_1 and its

Table 1
Effect of ligand binding on the percent distribution of CF_1 peptide hydrogens, with ρ values smaller than 10^{-4}, 10^{-5} and 10^{-6}

ρ Values	CF_1	ADP + CF_1	ϵATP + CF_1	ATP + CF_1
$0 < \rho < 10^{-4}$	28	35	35	46
$0 < \rho < 10^{-5}$	21	27	27	34
$0 < \rho < 10^{-6}$	15	21	21	24

adenine nucleotide complexes.

The amino acid analysis of CF_1 [10] indicates approximately 3000 potentially observable backbone NH groups. Under our experimental conditions, about 70% of the peptide NH groups of CF_1 have undergone exchange prior to any measurement (fig.2). Conversely, it appears that 15% of the peptide hydrogens have ρ smaller than 10^{-6} and must be buried in the interior of the protein or located at interfaces between subunits.

AMP had little effect on N^1H–N^2H exchange kinetics of CF_1 (fig.2). Only 2–3% of exchanging protons were affected but the reproducibility of a measurable effect is of the same order. In contrast, binding of ADP and ATP retarded the ^1H–^2H substitution (fig.2).

The exchange rate of the measurable hydrogens was reduced in the presence of ADP or ϵATP by a factor of 8 corresponding to a change in standard free energy of 4.8 kJ/mol for the conformational transition. ADP and ϵATP reduce the probability of solvent exposure of both fast and slow hydrogens in CF_1; 35% of the NH–groups in the complex instead of 28% in CF_1 have ρ values smaller than 10^{-4}, 27% instead of 21% have ρ values smaller than 10^{-5} and 21% instead of 15% have ρ values smaller than 10^{-6} (table 1). So, ADP or ϵATP prevents the exchange of 6–7% of peptide hydrogens, i.e., 180 to 210 NH residues.

CF_1 Exchange was reduced to the highest extent by ATP (fig.2). Rapidly and slowly exchanging hydrogens were differently affected (table 1): 46% of the peptide protons in the complex have ρ values smaller than 10^{-4} instead of 28% in the free enzyme; 24% instead of 15% have ρ values smaller than 10^{-6}, so that addition of ATP to CF_1 prevents the exchange of 9% additional hydrogens; i.e., 270 atoms by mole enzyme which are probably located in hindered hydrogen-bonded structures. The exchange rates in the ATP–CF_1 complex were retarded 20–35-fold, and the standard free energy difference was, on an average, 8 kJ/mol, suggesting a marked stabilizing effect of ATP upon dynamic CF_1 structures.

In conclusion, the conformation of the native enzyme appears to be looser than that of its adenine nucleotide complexes. Binding of ADP, ϵATP, or ATP to CF_1 is associated with a decreased probability of solvent-exposed peptide groups. Girault and Galmiche [8] found three sites of fixation of ADP/mol enzyme; however, it seems rather unlikely that three moles of nucleotide act by directly shielding so many hydrogens (180–270 NH) from water molecules. As CF_1 contains five different subunits, conformational changes induced by nucleotide binding may result in different interactions between the subunits. This rearrangement of the subunits may shield many protons from solvent exposure at their intersubunit contacts.

After fixation of ATP or ADP to the first nucleotide binding site, part of the peptide chain in the $\alpha\beta$ subunits of CF_1 is shielded from solvent effects, as indicated by the enhancement of the fluorescence of a probe, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole covalently bound to a NH_2 group of this peptide chain [8]; ADP induced this conformational change as effectively as ATP. The structural changes we have discussed in this paper are not similar after the addition of ATP or ADP to CF_1. This apparent discrepancy is likely to be CF_1 concentration dependent, as supported by infrared data. This investigation of the properties of concentrated solutions of CF_1 is very relevant, because CF_1 concentrations in vivo, if homogeneously distributed, should be 2×10^{-4} to 4×10^{-4} M.

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