Biochimica et Biophysica Acta 894 (1987) 127–137
Elsevier

BBA 42644

Ligand-induced conformational changes in the *Escherichia coli* F_1 adenosine triphosphatase probed by trypsin digestion

Philip D. Bragg and Cynthia Hou

Department of Biochemistry, University of British Columbia, Vancouver, B.C. (Canada)

(Received 21 April 1987)

Key words: ATPase, F_1-; Enzyme conformation; Trypsin digestion; Ligand binding; (*E. coli*)

Digestion of the F_1-ATPase of *Escherichia coli* with trypsin stimulated ATP hydrolytic activity and removed the δ and ε subunits of the enzyme. A species represented by the formula $\alpha_3^1\beta_3^1\gamma^1$, where α^1, β^1 and γ^1 are forms of the native α, β and γ subunits which have been attacked by trypsin, was formed by trypsin digestion in the presence of ATP. In the presence of ATP and $MgCl_2$, conversion of γ to γ^1 was retarded and the enzyme retained the ε subunit. These results imply that binding of ATP to the β subunits alters the conformation of ECF_1 to increase the accessibility of the γ subunit to trypsin. The likely trypsin cleavage sites in the α, β and γ subunits are discussed. ECF_1 from the α subunit-defective mutant *uncA401*, or after treatment with *N,N'*-dicyclohexylcarbodiimide or 4-chloro-7-nitrobenzofurazan, was present in a conformation in which the γ subunit was readily accessible to trypsin and could not be protected by the presence of ATP and $MgCl_2$. In a similar manner to native *E. coli* F_1-ATPase, the hydrolytic activity of the trypsin-digested enzyme was stimulated by the detergent lauryldimethylamine *N*-oxide. Since the digested enzyme lacked the ε subunit, a putative inhibitor of hydrolytic activity, a mechanism for the stimulation which involves loss or movement of this subunit is untenable.

Introduction

The cell membrane ATP synthase of *Escherichia coli* is similar in structure and properties to other energy-transducing ATP synthases [1–5]. It consists of an extrinsic membrane protein, the F_1-ATPase, having the active site(s) for ATP synthesis and hydrolysis. *E. coli* F_1-ATPase is composed of five subunits (α–ε) in a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$. The active sites of the enzyme are probably located on β subunits [6]. The α subunits have a binding site for ADP or ATP [7] and it is likely that the α subunits carry the tightly bound, non-catalytic adenine nucleotides which are found in the isolated *E. coli* F_1-ATPase [8,9]. The γ subunit has a role in organizing the structure of *E. coli* F_1-ATPase, and the δ and ε subunits bind the $\alpha_3\beta_3\gamma$ substructure to the membrane [10].

Net ATP synthesis and hydrolysis involves cooperative interactions between active sites. Mitochondrial F_1-ATPase (and *E. coli* F_1-ATPase) has a very low 'unisite' hydrolysis rate for ATP which is enhanced to a high 'multisite' rate when more than one catalytic site is occupied by sub-

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; NbfCl, 4-chloro-7-nitrobenzofurazan; IAANS, 2-(4'-iodoacetamidoanilino)naphthalene-6-sulfonic acid; LDAO, lauryldimethylamine *N*-oxide; DTT, dithiothreitol; DPCC-trypsin, trypsin treated with diphenylcarbamyl chloride to inhibit contaminating chymotrypsin; SDS, sodium dodecyl sulfate.

Correspondence: P.D. Bragg, Department of Biochemistry, University of British Columbia, 2146 Health Sciences Mall, Vancouver, Canada, V6T 1W5.

strate [11]. One approach to detect cooperativity between subunits has been by the use of aurovertin D, which binds specifically to the β subunits [12]. The fluorescence of bound aurovertin is markedly enhanced by nucleotides in normal *E. coli* F_1-ATPase, but not in the hydrolytically inactive *uncA401* ATPase [13,14]. Since α subunits of the mutant *E. coli* F_1-ATPase are defective [15], normal ATP hydrolysis must depend on cooperative interactions between the α and the catalytic β subunits.

Recently, we have described a further method to study the cooperative interactions between subunits [16–18]. Cysteinyl residues of *E. coli* F_1-ATPase can be labeled by a fluorescent reagent such as IAANS without affecting hydrolytic activity. Labeling of the single cysteinyl residues of the β subunits is prevented in *uncA401 E. coli* F_1-ATPase, thus confirming the cooperative interactions between α and β subunits [18]. The specific nature of these cooperative interactions between the subunits of *E. coli* F_1-ATPase is not known. Presumably, transient conformational changes are occurring during enzyme action.

In the present paper we have used trypsin digestion to explore the effects of ligand binding on the conformation of *E. coli* F_1-ATPase. Previous studies have indicated that trypsin digestion stimulates the hydrolytic activity of *E. coli* F_1-ATPase and that this is accompanied by loss of the δ and ε subunits, and by the removal of the 15 amino-terminal residues of the α subunits [19,20]. In this paper we show that the accessibility of a trypsin-cleavage site on the γ subunit is affected by the presence of Mg^{2+} and adenine nucleotides in the wild-type enzyme but not in the α-subunit-defective mutant *uncA401*. Treatment of the wild-type *E. coli* F_1-ATPase with DCCD or NbfCl, reagents which induce an alteration in the conformation of the enzyme as shown by changes in the accessibility of sulfhydryl groups [16,17], also affects the ability of ligands to protect the γ subunit from trypsin cleavage.

Materials and Methods

Strains

The following strains of *E. coli* were used: ML308-225 (wild-type), LE392pRPG54 (pRPG54 is Cm^r *uncB*$^+$*E*$^+$*F*$^+$*H*$^+$*A*$^+$*G*$^+$*D*$^+$*C*$^+$) [21] and AN120 (*uncA401*).

Preparation of E. coli F_1-ATPase

The F_1-ATPase were prepared as described previously [22] except that the fractions from the aminohexyl-Sepharose 4B column were applied to a sucrose gradient prepared in a buffer consisting of 0.1 M triethanolamine/0.5 mM EDTA/0.5 mM dithiothreitol, supplemented where indicated with 1 mM ATP, and adjusted to pH 7.5 with HCl.

Treatment of E. coli F_1-ATPase with trypsin

E. coli F_1-ATPase (1.5 mg) in 0.1 M triethanolamine/0.5 mM EDTA/0.5 mM dithiothreitol/20% (w/v) sucrose was treated with DPPC-trypsin (0.1 mg/ml) at 37 °C for the indicated time. The digestion was terminated by the addition of soybean trypsin inhibitor to a concentration of 0.4 mg/ml. This procedure was modified also by the inclusion of (a) 2.5 mM $MgCl_2$, (b) 5 mM ATP or ADP, (c) 5 mM ATP or ADP with 2.5 mM $MgCl_2$. In some instances the *E. coli* F_1-ATPase was freed of sucrose and/or ATP (if present) prior to trypsin treatment by use of centrifuged 1-ml columns of Sephadex G50 equilibrated with 50 mM triethanolamine-HCl (pH 7.5) containing 1 mM dithiothreitol [23].

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli [24]. Isoelectric focusing gels were run in one dimension using the O'Farrell system [25] modified as described in Ref. 26. For proteins with isoelectric points greater than 6.5 the buffer system of Thomas and Hodes [27], modified as before [26], was used. Proteins were stained with Coomassie blue [28] or detected by immunoblotting.

For immunoblotting, the polyacrylamide gel was incubated for 1 h with 50 mM Tris-HCl/20% (v/v) glycerol (pH 7.4). The proteins were transferred electrophoretically to nitrocellulose sheets (BioRad or Promega Biotec) in a BioRad Trans-Blot apparatus using a buffer of 10 mM $NaHCO_3$/3 mM Na_2CO_3/20% (v/v) methanol (pH 10). The transfer was carried out with water

cooling for 1 h at 0.85 A. The nitrocellulose sheet was treated for 30 min with 1% (w/v) bovine serum albumin (Promega Biotec) in 10 mM Tris-HCl/150 mM NaCl (pH 8), and subsequently for 30 min with the primary antibody, raised in rabbits, in Tris-NaCl. The binding of primary antibody was detected using goat antirabbit immunoglobulin coupled to alkaline phosphatase. Alkaline phosphatase activity was revealed using the 5-bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium reagent supplied by Promega Biotec.

Chemical treatments

E. coli F_1-ATPase was treated with NbfCl, DCCD or IAANS as described previously [17]. The Creighton modification of the sulfhydryl groups of ECF_1 with iodoacetic acid and iodoacetamide was carried out as described by Stan-Lotter and Bragg [26].

Assays

ATPase activity was measured at 37 °C in the presence of 2.5 mM $MgCl_2$ as described previously [19]. Protein was determined by the method of Bradford [29], with bovine serum albumin as a standard.

The fluorescence of aurovertin D in the presence of *E. coli* F_1-ATPase was measured at 20 °C in 2.0 ml of a buffer at pH 7.4 which contained *E. coli* F_1-ATPase (0.19 mg protein), 10 mM Tris-HCl, 0.25 M sucrose, 0.5 mM EDTA and 1.19 μM aurovertin D. Fluorescence was excited by light at 365 nm and emission was measured at 470 nm. Changes in fluorescence were measured following sequential additions of 10 μl 40 mM ADP, 100 μl 40 mM ATP and 10 μl 1.0 M $MgCl_2$ as described previously [14].

Chemicals

The following materials were supplied by the companies indicated. Sigma: DCCD, NbfCl, iodoacetic acid, iodoacetamide, DPPC-trypsin, soybean trypsin inhibitor. Molecular Probes Inc.: IAANS, sodium salt. Promega Biotec: nitrocellulose sheets, immuno-reagents. Onyx Chemical Company: LDAO. Aurovertin D was a gift from Dr. R.B. Beechey.

Results

Effect of trypsin digestion on subunits of wild-type E. coli F_1-ATPase

E. coli F_1-ATPase was treated with DPCC-trypsin in the presence or absence of 5 mM ATP or 5 mM ATP/2.5 mM $MgCl_2$ for various time intervals. Proteolytic digestion was terminated by the addition of soybean trypsin inhibitor and the products of digestion examined by SDS-polyacrylamide gel electrophoresis or on isoelectric focusing gels. As shown in Fig. 1, trypsin digestion stimulated the ATPase activity of *E. coli* F_1-ATPase, the stimulation being essentially complete within 5 min. In the absence of ligands, or with 5 mM ATP, enzyme activity was stimulated over 2-fold. There was less stimulation (50%) in the presence of ATP/$MgCl_2$. Examination of the products of digestion showed that all subunits had been digested to some extent (Fig. 2). There was a small decrease in the molecular weight of the α subunit. The β subunit migrated slightly more slowly on SDS-polyacrylamide gels. Careful examination suggested that at least two bands were present. The γ subunit progressively disappeared over the time course of the experiment. The δ and ε subunits were digested essentially completely by 5 min. The changes in the α, δ and ε subunits

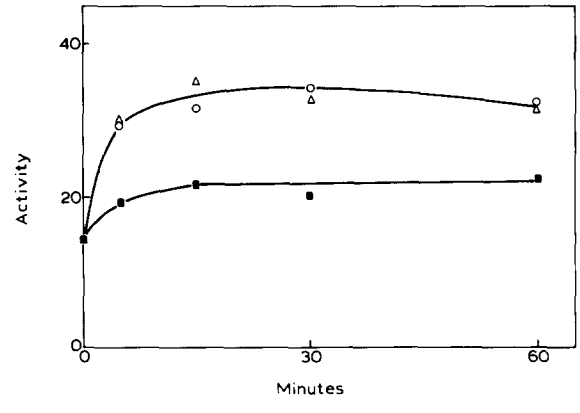

Fig. 1. Effect of trypsin on the activity of *E. coli* F_1-ATPase. *E. coli* F_1-ATPase was digested with DPCC-trypsin in the absence (circles) and presence of 5 mM ATP (triangles) or 5 mM ATP/2.5 mM $MgCl_2$ (squares) as described in Materials and Methods. Activity is expressed as specific activity. Also see Fig. 2.

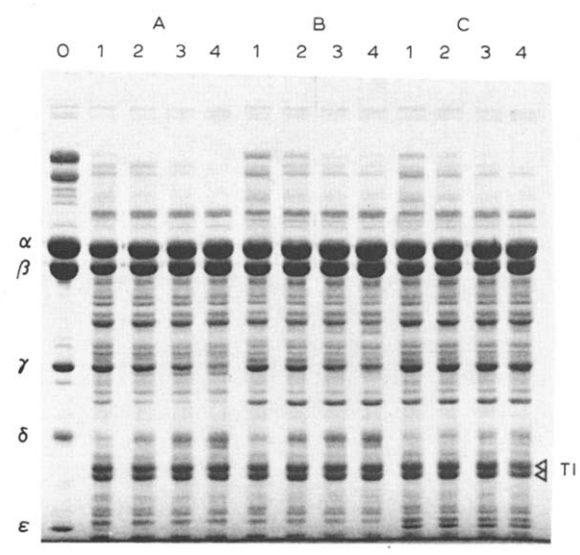

Fig. 2. Effect of trypsin on the subunits of *E. coli* F_1-ATPase. The samples were derived from the experiment shown in Fig. 1 and separated by SDS-polyacrylamide gel electrophoresis. 0, untreated enzyme; 1–4, samples removed at 5, 15, 30 and 60 min, respectively. *E. coli* F_1-ATPase was digested in the absence (A) and presence of 5 mM ATP (B) or 5 mM ATP/2.5 mM $MgCl_2$ (C). The positions of migration of the $\alpha-\varepsilon$ subunits of *E. coli* F_1-ATPase are indicated. TI, trypsin inhibitor.

most closely paralleled the time-course for the stimulation of ATPase activity (Fig. 1).

The results described above indicated that trypsin treatment in the presence of 5 mM ATP accelerated the digestion of the γ subunit whereas 5 mM ATP/2.5 mM $MgCl_2$ protected the γ subunit and gave retention of the ε subunit (Fig .2). These effects could be seen more clearly following reseparation of the digested enzymes on a sucrose gradient (Fig. 3). ADP (5 mM)/2.5 mM $MgCl_2$ was as effective as ATP/$MgCl_2$ in protecting the γ subunit (Fig. 3(A, B)). Inclusion of 2.5 mM $MgCl_2$ alone, like ATP, accelerated the rate of digestion of the γ subunit (results not shown). The effect of these ligands on the digestion of the α and β subunits is shown in Fig. 3(C, D). Except for the generation of slightly faster migrating species of the α subunit, presumably by removal of the amino-terminal 15 amino acids [20], trypsin digestion had little effect on this subunit. Inclusion of ATP and/or $MgCl_2$ had no discernible effect on the digestion of the α subunit. The changes with the β subunit were more complex.

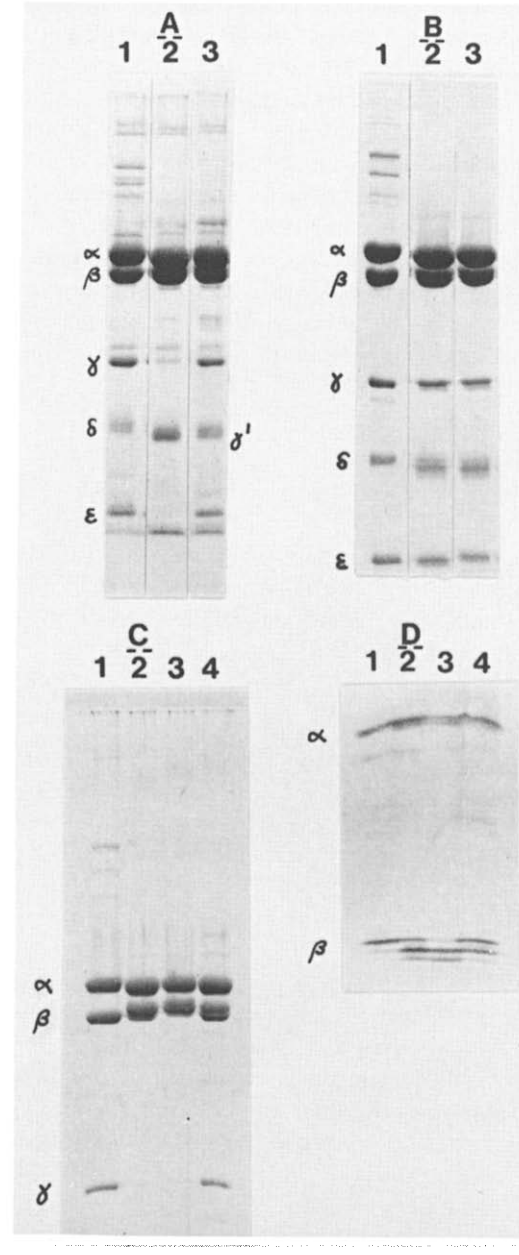

Fig. 3. Effect of nucleotides and Mg^{2+} on the digestion of the subunits of *E. coli* F_1-ATPase by trypsin. The enzyme was reseparated on a sucrose gradient after treatment. Samples were examined by SDS-polyacrylamide gel electrophoresis (A–C) or by isoelectric focusing (D). The positions of migration of the $\alpha-\varepsilon$ subunits of *E. coli* F_1-ATPase are indicated. *E. coli* F_1-ATPase was digested with DPCC-trypsin as described in Materials and Methods in the presence or absence of the following components: (A) 1, no ATP or $MgCl_2$; 2, 5 mM ATP; 3, 5 mM ATP/2.5 mM $MgCl_2$. (B) 1, no ADP or $MgCl_2$; 2, 5 mM ADP; 3, 5 mM ADP/2.5 mM $MgCl_2$. (C) and (D) 1, untreated *E. coli* F_1-ATPase; 2, no ATP or $MgCl_2$; 3, 2.5 mM $MgCl_2$; 4, 5 mM ATP/2.5 $mgCl_2$.

The stained band of the β subunit on SDS-polyacrylamide gels was clearly composed of several incompletely separated species migrating more slowly than the undigested subunit (Fig. 3(C)). Digestion in the presence of ATP/$MgCl_2$ gave almost equal amounts of undigested β subunit and a cleavage product which had a position of migration on IEF gels equivalent to the net loss of a single positive charge from the native subunit (Fig. 3(D,4)). Digestion in the absence of ligands showed a greater formation of this species (Fig. 3(D,2)), together with a new species migrating in a position on IEF gels equivalent to the net loss of two positive charges. In the presence of $MgCl_2$ little of the β subunit remained undigested. The two new species observed above were present (Fig. 3(D,3)).

The digestion of the γ subunit was accompanied by the appearance of a new band migrating close to the position of the δ subunit on SDS-polyacrylamide gel electrophoresis. The molecular mass of the fragment (γ^1) was about 20 kDa. The relationship of γ^1 to the γ subunit was confirmed by immunoblotting (Fig. 4). Samples of *E. coli* F_1-ATPase were treated with trypsin in the absence and presence of 5 mM ATP, and the products of digestion examined by SDS-polyacrylamide gel electrophoresis. The products were transferred electrophoretically onto nitrocellulose sheets and treated with polyclonal rabbit antibodies against the γ, δ and ε subunits. Binding of antibody to the subunit or subunit fragment was recognized with goat antirabbit immunoglobulin coupled to alkaline phosphatase. As shown in Fig. 4(A), the presence of ATP accelerated the digestion of the γ and ε subunits. The γ^1 band which increased in intensity in the region of the δ subunit was clearly derived from the γ subunit (Fig. 4(B)). The δ subunit was removed completely by trypsin digestion (Fig. 4(C)).

The γ subunit contains two cysteine residues [30] which may be readily demonstrated using the Creighton procedure [26]. This method is based on the following consideration. By reacting cysteinyl-containing denatured and reduced polypeptides with iodoacetate acidic carboxymethyl groups are introduced, while reaction with neutral iodoacetamide causes no charge differences. By using varying ratios of iodoacetamide to iodoacetate a spectrum of polypeptide molecules is generated having 0–n acidic carboxymethyl groups, where n is the number of cysteinyl residues per molecule of polypeptide. When these species are separated on isoelectric focusing gels, $n + 1$ species will be seen after staining for protein. Thus, an integral number of cysteinyl groups can be determined by counting the bands. Application of this method showed that the γ^1 fragment produced on trypsin digestion of *E. coli* F_1-ATPase retained the two cysteine residues of the native subunit (Fig. 5).

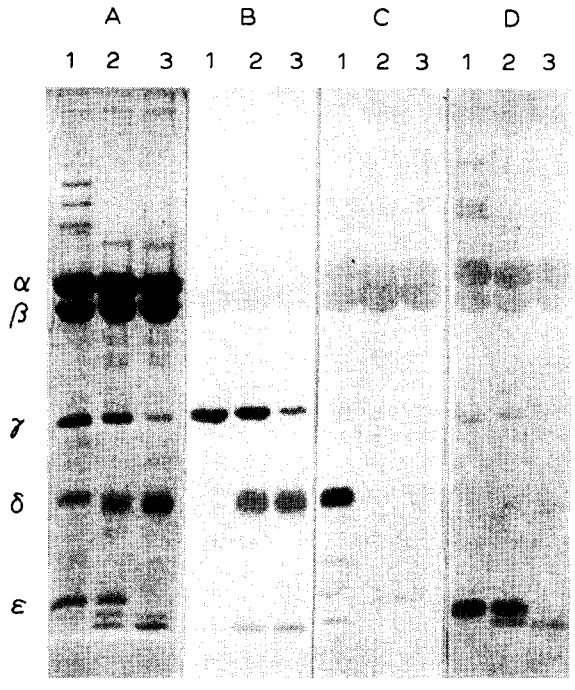

Fig. 4. Examination of the products of trypsin digestion of *E. coli* F_1-ATPase by immunoblotting. *E. coli* F_1-ATPase was digested by DPCC-trypsin in the absence (2) or presence of 5 mM ATP (3), and reseparated on a sucrose gradient. The products of digestion were examined by SDS-polyacrylamide gel electrophoresis. 1, untreated enzyme. The gel was stained with Coomassie blue (A) or the separated subunits transferred electrophoretically to nitrocellulose (B–D). The transferred subunits, and their digestion products, were detected using antisera raised against the γ (B), δ (C) and ε (D) subunits. The procedures are described in Materials and Methods. The positions of migration of the α–ε subunits of *E. coli* F_1-ATPase are indicated.

Properties of trypsin-digested E. coli F_1-ATPase

The gels of Figs. 3 and 4 use *E. coli* F_1-ATPase

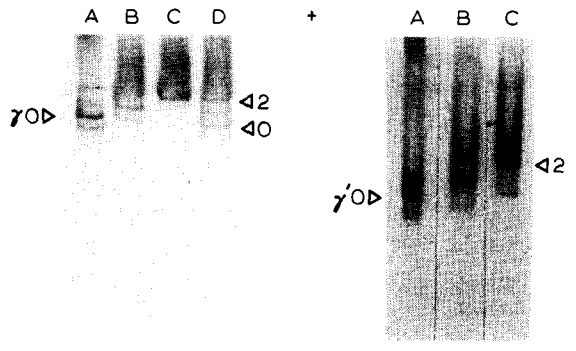

Fig. 5. Creighton modification of native and trypsin-treated *E. coli* F_1-ATPase with iodoacetamide and iodoacetate. The positions of migration of unlabeled γ and γ^1 subunits are shown. The figures at the right indicate the number of charges introduced by reaction of the sulfhydryl groups with iodoacetate. The gel system of Thomas and Hodes [27] was used. Lanes A, iodoacetamide; B, iodoacetamide/iodoacetate (1 : 5); C, iodoacetate; D, mixture of A–C.

which had been treated with trypsin in the presence of ATP or ATP/$MgCl_2$ and then reseparated on a sucrose gradient. The treated enzyme sedimented to a position in the gradient similar to the untreated enzyme. As can be seen from these gels, the γ^1 fragment remains associated with the α and β subunits. The specific activity of the *E. coli* F_1-ATPase treated with trypsin in the presence of 5 mM ATP (57.8 μmol/min per mg protein) was about twice that of the untreated enzyme (34.1) or of the enzyme which had been digested in the presence of 5 mM ATP/2.5 mM $MgCl_2$ (37.5). The increase in activity is presumably associated with the loss of the ε subunit, which is known to inhibit ATPase activity [10]. The enzyme treated in the presence of ATP or ATP/$MgCl_2$ retained the ability of the native *E. coli* F_1-ATPase to be stimulated 2–3-fold by inclusion of 0.5% LDAO in the assay mixture. The guanosine and inosine triphosphatase activities of the treated enzymes were similar to those of the native *E. coli* F_1-ATPase.

We have shown previously that the single sulfhydryl groups of β subunits are labeled preferentially over the sulfhydryl groups of α subunits by certain fluorescent iodoacetamide-based reagents [16]. Treatment of native *E. coli* F_1-ATPase with IAANS preferentially labeled β, γ, δ and ε subunits (results not shown), the preferential labeling of the β subunits over a α subunits was retained in the enzyme treated with trypsin in the presence of ATP or ATP/$MgCl_2$. At least one of the sulfhydryl groups of the γ^1 fragment was labeled by IAANS.

Changes in the fluorescence of *E. coli* F_1-ATPase-bound aurovertin D on addition of adenine nucleotides and $MgCl_2$ have been used to demonstrate conformational changes in the enzyme [13,14]. A typical response of the native enzyme is shown in Fig. 6 (left panel). Micromolar concentrations of ADP enhanced the fluorescence of the bound aurovertin. This enhancement was decreased by addition of 10-fold higher levels of ATP, and obliterated by $MgCl_2$. *E. coli* F_1-ATPase which had been digested in the presence of 5 mM ATP/2.5 mM $MgCl_2$ behaved similarly (Fig. 6, right panel), presumably because of the partial protection afforded by these ligands during digestion. The fluorescence response to the addition of nucleotides and $MgCl_2$ of the enzyme which had been digested in the presence of 5 mM ATP was very small (Fig. 6, centre panel). However, in this case there was a greater than usual enhancement in the fluorescence of aurovertin when it bound to the enzyme in the absence of ligands. The negligible extent of the response of the di-

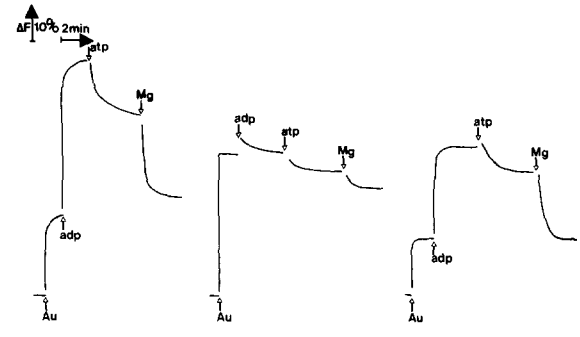

Fig. 6. Effect of ADP, ATP and Mg^{2+} on the fluorescence intensity of aurovertin D-ATPase complex. Fluorescence measurements were as described in Materials and Methods. Where indicated, 2.38 nmol, 0.4 μmol, 4 μmol and 10 μmol of aurovertin D (Au), ADP (adp), ATP (atp) and $MgSO_4$ (Mg) were added, respectively. Left panel: untreated *E. coli* F_1-ATPase; centre panel, *E. coli* F_1-ATPase treated with DPCC-trypsin in the presence of 5 mM ATP; right panel: *E. coli* F_1-ATPase, treated with DPCC-trypsin in the presence of 5 mM ATP/2.5 mM $MgCl_2$. All enzymes were reseparated on a sucrose gradient after treatment.

gested enzyme to added ligands in interesting when compared with the higher than usual hydrolytic activity of the treated enzyme.

Trypsin digestion of mutant E. coli F_1-ATPase

The *E. coli* F_1-ATPase of the *uncA401* strain has a replacement of serine 373 of the α subunits by phenylalanine [15]. It is hydrolytically inactive. As with the wild-type enzyme, digestion of the *uncA401* enzyme resulted in loss of δ and ε subunits, and conversion of γ to the γ^1 fragment. However, the presence of 5 mM ATP/2.5 mM $MgCl_2$ (or $MgCl_2$ and ATP alone) did not protect γ and ϵ subunits from digestion (Fig. 7). IEF gels (Fig. 7) revealed that the β subunit was cleaved to give the product migrating with the net loss of one positive charge that was observed in the wild-type enzyme (Fig. 5). ATP and ATP/$MgCl_2$ could not protect the β subunit from cleavage. It is of interest to note that $MgCl_2$ increased the extent of cleavage of the β subunit to the product with two less positive charges (Fig. 7). Thus, the mutant enzyme has some capacity to respond to the presence of ligands.

Trypsin digestion of NbfCl- and DCCD-modified E. coli F_1-ATPase

Reaction of F_1-ATPase with NbfCl or DCCD inhibits ATPase activity through modification of tyrosyl or carboxyl groups in β subunits [31,32]. As shown in fig. 8, for NbfCl, inhibition of ATPase activity by these reagents was associated with the loss of the ability of ATP/$MgCl_2$ to protect γ and ε subunits from digestion by trypsin. Digestion of α, β and δ subunits followed that established for the wild-type enzyme.

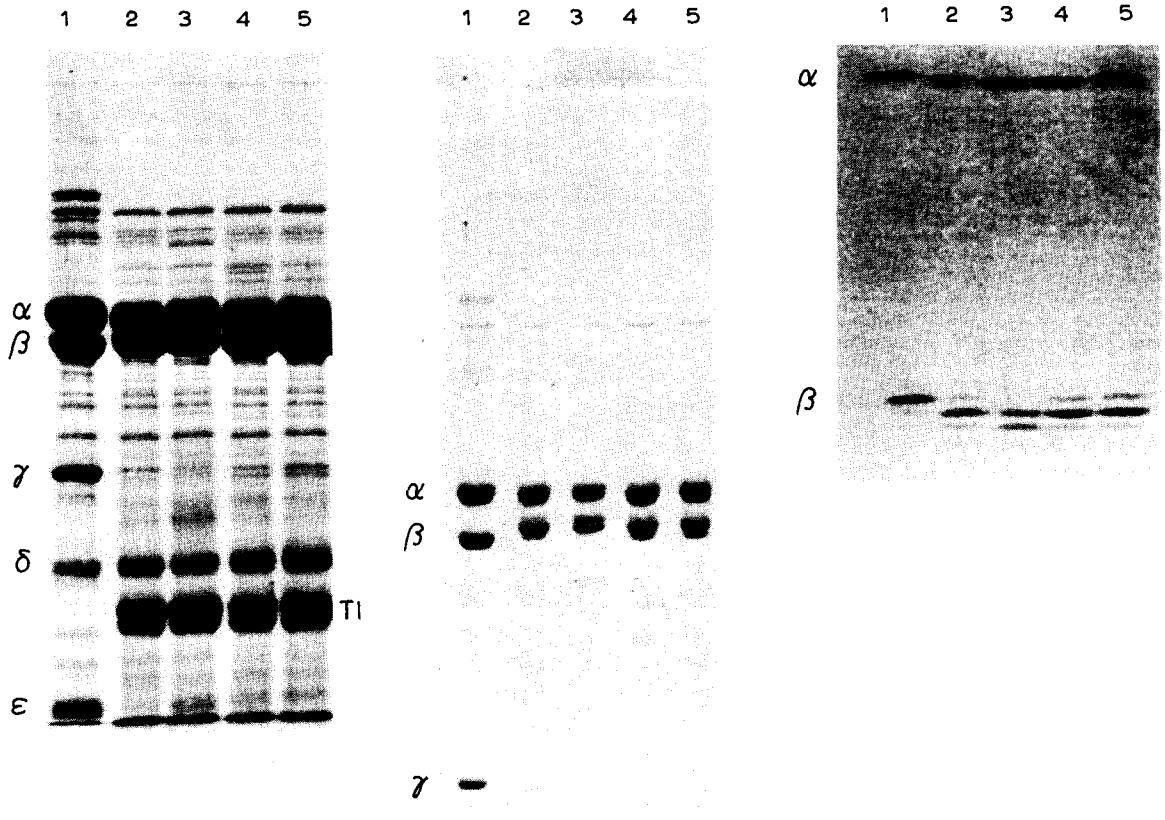

Fig. 7. Effect of trypsin on the subunits of *E. coli* F_1-ATPase from the *uncA401* mutant. The enzyme was untreated (1), or treated with trypsin in the absence of ligands (2) or in the presence of 2.5 mM $MgCl_2$ (3), 5 mM ATP (4) and 5 mM ATP/2.5 mM $MgCl_2$ (5). The subunits were resolved by SDS-polyacrylamide gel electrophoresis (left and centre panels) or by isoelectric focusing (right panel). The positions of migration of the α–ε subunits of *E. coli* F_1-ATPase and of trypsin inhibitor (TI) are indicated.

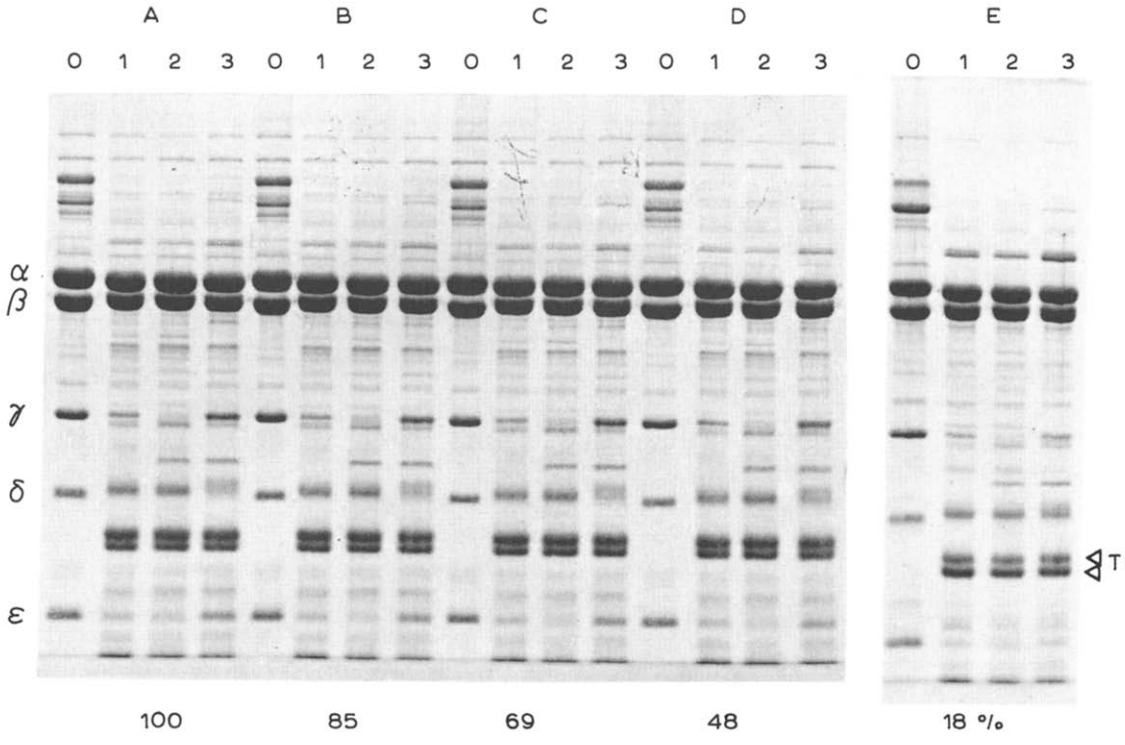

Fig. 8. Effect of NbfCl treatment of *E. coli* F_1-ATPase on the digestion of the subunits by trypsin. *E. coli* F_1-ATPase was treated with NbfCl for 0 (A), 5 (B), 15 (C), 30 (D) or 60 (E) min as described in Materials and Methods. The NbfCl-treated enzymes were digested with DPCC-trypsin in the absence (1) and presence of 5 mM ATP (2) or 5 mM ATP/2.5 mM $MgCl_2$ (3), or not digested (0). The products of digestion were examined by SDS-polyacrylamide gel electrophoresis. The positions of migration of the α–ε subunits and of trypsin inhibitor (TI) are indicated. The percentage of the ATPase activity remaining at each time interval during NbfCl treatment is shown.

Discussion

Previous studies [19,20] of the effect of trypsin on *E. coli* F_1-ATPase showed that the δ and ε subunits were readily digested, that 15 amino acids were removed from the amino-terminus of the α subunits, and that the β subunits were apparently unattacked. The γ subunit was digested to a variable degree, but no distinct digestion product of it was recognized. Loss of δ was associated with the inability of the enzyme to rebind to membranes. It is likely that δ was lost from the trypsin-treated enzyme, because it interacted with the terminal amino-acid sequence removed by trypsin from the α subunit. Finally, trypsin digestion resulted in stimulation of ATPase activity.

In the present paper we have found that trypsin treatment of *E. coli* F_1-ATPase in the presence of ATP generated an active form of the ATPase, lacking δ and ε subunits, which sedimented in a sucrose gradient similarly to the native enzyme. The form of enzyme can be represented by the formula $\alpha_3^1\beta_3^1\gamma^1$, where α^1, β^1 and γ^1 are α, β and γ subunits which have been attacked by trypsin. The presence of ATP with $MgCl_2$ prevented the conversion of γ to γ^1 and resulted in the retention of the ε subunit. These results imply that the conformation of *E. coli* F_1-ATPase must be different in the presence of ATP/$MgCl_2$ compared with ATP alone, and that these conformations differ in the extent of exposure of the γ subunit to the medium. The sites of trypsin cleavage of the subunits have not been established unambiguously. Dunn [20] showed that trypsin removed the amino-terminal 15 amino acids of the α subunit. We have found, contrary to previous indications [20,33], that the β subunit of *E. coli* F_1-ATPase was cleaved by trypsin. The number of amino

acids removed from this subunit must be small since there was little apparent change in molecular weight. Two cleavage products were observed, being successively one net charge less positive than the undigested β subunit (Fig. 3). Although the sites of cleavage have not been identified, examination of the amino acid sequence of the β subunit [30] suggests that one cleavage site could be at lysine-5. This would generate a residual species of the appropriate net charge. A trypsin cleavage site at lysine-7 of the β subunit of the bovine mitochondrial F_1 has been observed by Walker et al. [34]. A second cleavage site close to the amino terminus of the β subunits of *E. coli* F_1-ATPase is unlikely as there would be an inappropriate change in charge. Therefore, a second cleavage site likely results in the removal of the terminal dipeptide from the carboxyl terminus of the β subunit. This would generate a β fragment of the appropriate charge. An alternative explanation is that trypsin removes the carboxyl-terminal dipeptide followed by the removal of a second dipeptide from this end of the β subunit molecule. Binding of Mg^{2+} to the enzyme makes the second cleavage site more accessible to the protease. A further implication of the pattern of trypsin digestion is that the amino-terminal region of the α subunit, and the carboxyl-terminal region of the β subunit, are probably at the surface of *E. coli* F_1-ATPase.

A preliminary attempt was made to identify the cleavage site(s) leading to the approx. 20000 Da fragment (γ^1) of the γ subunit. The Creighton modification procedure [26] demonstrated that γ^1 still retained the two cysteine residues of the γ subunit. Thus, removal of about 10 kDa of amino acids (i.e., the difference in molecular mass of γ and γ^1) cannot occur by removal of a 10 kDa amino-terminal fragment, as this would result in the loss of a cysteine residue from γ^1. Moreover, the fragment γ^1 was much more basic than the γ subunit, as shown by its behaviour on IEF gels (Fig. 5). The γ subunit has a predominance of basic over acidic amino acid residues (19 : 9) in the amino-terminal 100 amino acids and the reverse holds for the carboxyl-terminal 100 amino acids (10 : 15) [30]. This means that removal of an amino-terminal fragment of 10 kDa would generate a more acidic fragment. This is contrary to what was found. However, removal of a 10 kDa segment from the carboxyl terminus would leave both cysteinyl residues in the γ^1 fragment and the γ^1 would be more basic than the γ subunit, as was observed. Thus, it is likely that trypsin cleaves the γ subunit in the carboxyl-terminal one-third of the molecule. This region of the subunit must be accessible to the medium, and its accessibility be altered by the binding of ATP and $MgCl_2$ to the enzyme. An alternative explanation is that the ε subunit protects the cleavage site on the γ subunit, and that these ligands reduce the extent of digestion of the ε subunit.

It is difficult to interpret the difference in effect of ATP and ATP/$MgCl_2$ on the conformation of *E. coli* F_1-ATPase. There are three ATP-binding sites on the enzyme, probably on β subunits [4,6]. In the most likely mechanism for the ATP hydrolysis each site would act in sequence [35]. However, there could be separate sites for ATP hydrolysis and for enzyme regulation [36]. In the latter case ATP alone might be binding to a regulatory site whereas in the presence of Mg^{2+} the catalytic site would be filled by the enzyme substrate MgATP. The effect of ATP and ATP/$MgCl_2$ on trypsin digestion has features in common with the action of these ligands on the fluorescence of aurovertin D bound to *E. coli* F_1-ATPase. As shown in Fig. 6, micromolar concentrations of ADP (or ATP) added to native *E. coli* F_1-ATPase caused a great enhancement of the fluoresence of the aurovertin. This enhancement was decreased to some extent by addition of ATP at higher concentrations, and discharged completely by Mg^{2+} [14]. A minimum of two conformational forms of *E. coli* F_1-ATPase can be inferred from these data: a low fluorescent form present in the absence of added ligands, or when Mg^{2+} or ATP (ADP)/Mg^{2+} are present, and a highly fluorescent form produced by micromolar concentrations of ADP or ATP. (Higher concentrations of ATP may produce a third, highly fluorescent form.) The highly fluorescent form would be the conformation in which the γ subunit of *E. coli* F_1-ATPase is accessible to trypsin. The γ subunit would be protected from trypsin action in the low-fluorescent form.

It was of interest to examine the effect on the trypsin digestion pattern of mutations in the enzyme and of chemical modifications which are

known to affect conformational interactions in *E. coli* F_1-ATPase. In the enzyme from the *uncA401* mutant serine-373 of α subunits is replaced by phenylalanine [15]. This does not prevent binding of adenine nucelotides but cooperative interactions between α and β subunits are impaired, as shown by modified aurovertin fluorescence behavior [13,14] and by the inhibition of the labeling by IAANS of the cysteine residues on the β subunit [18]. Trypsin treatment of the *uncA401* ATPase resulted in complete conversion of the γ subunit to γ^1. This reaction could not be prevented by ATP/$MgCl_2$ (Fig. 7). Thus, the mutant *E. coli* F_1-ATPase must be present in a conformation in which the γ subunit is accessible to trypsin, and the lack of cooperative interactions between subunits prevents the enzyme from responding to the binding of certain ligands. However, the enzyme did show some response to Mg^{2+}. In the presence of these ions there was a greater conversion of β subunits to the doubly cleaved product. Perhaps this indicates the presence of a Mg^{2+}-binding site on the β subunits.

NbfCl and DCCD modify the β subunits of *E. coli* F_1-ATPase with inhibition of ATPase activity [31,37]. This modification alters the conformation of *E. coli* F_1-ATPase so that the cysteinyl residues on α subunits are now labeled preferentially over β subunits, as found with the wild-type enzyme [16–18]. The alteration in the conformation of *E. coli* F_1-ATPase by these inhibitors was reflected in the trypsin-digestion behavior. The γ subunits of the NbfCl- and DCCD-modified enzymes were accessible to trypsin digestion and ATP/$MgCl_2$ did not protect them.

The results described above indicate that trypsin digestion is a useful probe of adenine nucleotide- and Mg^{2+}-induced conformational states of *E. coli* F_1-ATPase. A similar conclusion was reached by Di Pietro et al. [38] for the mitochondrial F_1, although there are differences in the patterns of digestion between the bacterial and mitochondrial enzymes. Khananshvili and Gromet-Elhanan [39] have used trypsin to show that binding of adenine nucleotide changes the conformation of the isolated β subunit of the F_1 of *Rhodospirillum rubrum*.

A further point to emerge from the studies described in this paper is that the adenine nucleotide-induced conformational changes in *E. coli* F_1-ATPase monitored by aurovertin fluorescence were lost on trypsin digestion, whereas the hydrolytic activity of the enzyme was unimpaired. Since the aurovertin bound to the digested enzyme to a greater extent, or with a greater fluorescence yield, trypsin digestion must have impaired the transmission to the aurovertin binding site of the conformational change which occurred on binding of nucleotide. However, trypsin digestion of *E. coli* F_1-ATPase did not affect its conformation so as to prevent the preferential labeling of the cysteinyl residues of the β subunits by IAANS [16] or to inhibit stimulation of its ATPase activity by LDAO. Stimulation by LDAO occurred in the absence of the ε subunit. Thus, the ε subunit cannot have a role in LDAO stimulation, as has been proposed [40].

Acknowledgements

This work was supported by a grant from the Medical Research Council of Canada. We thank Dr. R.D. Simoni (Stanford University) for a gift of the antibodies against the subunits of *E. coli* F_1-ATPase.

References

1 Futai, M. and Kanazawa, H. (1983) Microbiol. Rev. 47, 285–312
2 Senior, A.E. and Wise, J.G. (1983) J. Membrane Biol. 73, 105–124
3 Vignais, P.V. and Satre, M. (1984) Mol. Cell Biochem. 60, 33–70
4 Bragg, P.D. (1984) Can. J. Biochem. Cell Biol. 62, 1190–1197
5 Walker, J.E., Saraste, M. and Gay, N.J. (1984) Biochim. Biophys. Acta 768, 164–200
6 Issartel, J.P., Lunardi, J. and Vignais, P.V. (1986) J. Biol. Chem. 261, 895–901
7 Dunn, S.D. and Futai, M. (1980) J. Biol. Chem. 255, 113–118
8 Bragg, P.D. and Hou, C. (1977) Arch. Biochem. Biophys. 178, 486–494
9 Maeda, M., Futai, M. and Anraku, Y. (1977) Biochem. Biophys. Res. Commun. 75, 331–338
10 Dunn, S.D. and Heppel, L.A. (1981) Arch. Biochem. Biophys. 210, 421–436
11 Grubmeyer, C., Cross, R.L. and Penefsky, H.S. (1982) J. Biol. Chem. 257, 12092–12100
12 Issartel, J.P., Klein, G., Satre, M. and Vignais, P.V. (1983) Biochemistry 22, 3492–3497

13 Wise, J.G., Latchney, L.R. and Senior, A.E. (1981) J. Biol. Chem. 256, 10383–10389
14 Bragg, P.D., Stan-Lotter, H. and Hou, C. (1982) Arch. Biochem. Biophys. 213, 669–679
15 Noumi, T., Futai, M. and Kanazawa, H. (1984) J. Biol. Chem. 259, 10076–10079
16 Stan-Lotter, H. and Bragg, P.D. (1986) Eur. J. Biochem. 154, 321–327
17 Stan-Lotter, H. and Bragg, P.D. (1986) Arch. Biochem. Biophys. 248, 116–120
18 Stan-Lotter, H. and Bragg, P.D. (1986) Eur. J. Biochem. 160, 169–174
19 Bragg, P.D. and Hou, C. (1975) Arch. Biochem. Biophys. 167, 311–321
20 Dunn, S.D., Heppel, L.A. and Fullmer, C.S. (1980) J. Biol. Chem. 255, 6891–6896
21 Gunsalus, R.P., Brusilow, W.S.A. and Simoni, R.D. (1982) Proc. Natl. Acad. Sci. USA 79, 320–324
22 Bragg, P.D. and Hou, C. (1986) Arch. Biochem. Biophys. 244, 361–372
23 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899
24 Laemmli, U.K. (1975) Nature 227, 680–685
25 O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007–4021
26 Stan-Lotter, H. and Bragg, P.D. (1984) Arch. Biochem. Biophys. 229, 320–328
27 Thomas, J.M. and Hodes, M.E. (1981) Anal. Biochem. 118, 194–196
28 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
29 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
30 Walker, J.E., Gay, N.J., Saraste, M. and Eberle, A.N. (1984) Biochem. J. 224, 799–815
31 Yoshida, M., Allison, W.S., Esch, F.S. and Futai, M. (1982) J. Biol. Chem. 257, 10033–10037
32 Andrews, W.W., Hill, F.C. and Allison, W.S. (1984) J. Biol. Chem. 259, 8219–8225
33 Bragg, P.D. and Hou, C. (1978) Can. J. Biochem. 56, 559–564
34 Walker, J.E., Fearnley, I.M., Gay, N.J., Gibson, B.W., Northrop, F.D., Powell, S.J., Runswick, M.J., Saraste, M. and Tybulewicz, V.L.J. (1985) J. Mol. Biol. 184, 677–701
35 Gresser, M.J., Meyers, J.A. and Boyer, P.D. (1982) J. Biol. Chem. 257, 12030–12038
36 Wang, J.H. (1986) J. Bioenerget. Biomemb. 18, 101–111
37 Lunardi, J., Satre, M., Bof, M. and Vignais, P.V. (1979) Biochemistry 18, 5310–5316
38 Di Pietro, A., Godinot, C. and Gautheron, D.C. (1983) Biochemistry 22, 785–792
39 Khananshvili, D. and Gromet-Elhanan, Z. (1986) Biochemistry 25, 6139–6144
40 Lötscher, H.-R., De Jong, C. and Capaldi, R.A. (1984) Biochemistry 23, 4140–4143