Met23Lys mutation in subunit gamma of F_OF_1-ATP synthase from *Rhodobacter capsulatus* impairs the activation of ATP hydrolysis by protonmotive force.

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SUMMARY

H^+-F_OF_1-ATP synthase couples proton flow through its membrane portion, F_O, to the synthesis of ATP in its headpiece, F_1. Upon reversal of the reaction the enzyme functions as a proton pumping ATPase. Even in the simplest bacterial enzyme the ATPase activity is regulated by several mechanisms, involving inhibition by MgADP, conformational transitions of the ε subunit, and activation by protonmotive force. Here we report that the Met23Lys mutation in the γ subunit of the *Rhodobacter capsulatus* ATP synthase significantly impaired the activation of ATP hydrolysis by protonmotive force. The impairment in the mutant was presumably due to the faster enzyme deactivation that was particularly evident at low ATP/ADP ratio. We suggest that the electrostatic interaction of the introduced γLys23 with the DELSEED region of subunit β stabilized the ADP-inhibited state of the enzyme by hindering the rotation of subunit γ rotation which is necessary for the activation.

146 words.

INTRODUCTION

H^+ transporting F_OF_1-ATP synthase (F_OF_1-complex) catalyses ATP synthesis/hydrolysis that is coupled to transmembrane proton transport. F_OF_1 is present in the inner membranes of mitochondria, thylakoid membranes of chloroplasts and bacterial plasma membranes. Enzymes from different organisms show strikingly high structural and functional homology and presumably have the same catalytic mechanism.

F_OF_1-ATP synthase is composed of two distinct portions connected by two "stalks". The hydrophilic F_1-portion (in the simplest bacterial enzyme a complex of five types of subunits in stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$) protrudes by ~100 Å from the membrane and is responsible for ATP synthesis/hydrolysis. The larger α and β subunits form a hexamer with elongated subunit γ inside it. The hydrophobic F_O-portion (in most bacteria a complex of three types of subunits in stoichiometry $a_1b_2c_{\sim 10}$) is embedded into the membrane and translocates protons. One of the two stalks mentioned above is composed by centrally located γε-subunits complex bound to *c*-subunits oligomer. Another one is formed by peripheral b_2-dimer (*bb'* heterodimer in case of *Rhodobacter capsulatus*) that connects subunit *a* to the $\alpha_3\beta_3\delta$-complex (see [1-4] for reviews on the F_OF_1 structure).

A unique feature of the enzyme is the rotary catalysis [5-7]. During ATP synthesis proton transport through F_O drives the rotation of $\gamma_1\varepsilon_1c_{\sim 10}$-complex (so called "rotor") relative to the rest of the enzyme, or "stator" $\alpha_3\beta_3\delta_1a_1b_2$ (see [8-13] for recent reviews).

The details of energy transmission between the catalytic F_1-portion and the proton transporting F_O are not fully understood. One of the main reasons for that is the complex regulation of the ATP synthase. A well-known regulatory feature of ATP synthase is inhibition of its ATPase activity by ADP. It is demonstrated that the binding (or failure to release) of MgADP at the high affinity catalytic site inactivates the enzyme in terms of ATP hydrolysis [14-21]. Upon the energization of the membrane, the tightly bound ADP is

released from the F_1-portion [22-25]. Several studies on bacterial, chloroplast and mitochondrial F_OF_1 have shown that after membrane energization the ATPase activity of the enzyme increased markedly [26-32], suggesting that release of the tightly bound ADP relieves the inhibition.

In this work we have further investigated the activation of ATP hydrolysis in F_OF_1 of the photoheterotrophic bacteria *Rhodobacter capsulatus* that was induced by transmembrane proton electrochemical potential difference ($\Delta\tilde{\mu}_{H^+}$). Isolated membrane vesicles (chromatophores) derived from these bacteria contain complete photosynthetic electron transport chain and F_OF_1. The important advantages of chromatophores are: 1) $\Delta\tilde{\mu}_{H^+}$ can be generated by light; transmembrane voltage ($\Delta\psi$) jumps of up to ≈100 mV can be achieved in a few milliseconds if a short flash of light is used for excitation; 2) voltage transients and thereby transmembrane charge transfer can be monitored with high time resolution by the electrochromic absorption band shift of intrinsic carotenoid pigments [33;34]; 3) the electrical ($\Delta\psi$) or the chemical (ΔpH) components of the $\Delta\tilde{\mu}_{H^+}$ can be selectively switched off by appropriate ionophores; 4) it is possible to prepare very small chromatophore vesicles (average diameter of approximately 30 nm) [34] that contain less than one active F_OF_1 per vesicle on average, which allows a "single molecule per vesicle" study [34;35].

Taking advantage of these favorable features, we investigated the activation of ATP hydrolysis in *Rb. capsulatus* wild-type F_OF_1 and in the mutated enzyme with γMet23 changed to Lys.

This mutation has been studied previously in the *E. coli* enzyme where it was shown to affect coupling between ATP hydrolysis and proton transport, while slightly impairing catalysis [36;37]. The mutation was proposed to introduce extra electrostatic interactions between γLys23 and βGlu381 in the ^{380}DELSEED^{386} segment of the β subunit [38;39].

However, the ATP induced rotation of γ-subunit in the purified F_1-portion (as detected with an attached actin filament providing a heavy viscous load) was undistinguishable in the mutant and in the wild-type enzyme [40]. The author concluded that the uncoupling was likely to occur at the interface between F_1 and F_O.

In this work we report that the activation of ATP hydrolysis by $\Delta\tilde{\mu}_{H^+}$ was severely impaired in the mutant enzyme. To our knowledge, this is the first experimental demonstration that a single amino acid substitution might affect such activation. Our data indicate that the rotation of subunit γ might play an important role in activation of ATP hydrolysis by $\Delta\tilde{\mu}_{H^+}$.

Materials and Methods

Cell growth and chromatophores preparation

Rb. capsulatus B100 strain was grown photoheterotrophically in a synthetic medium (RCV medium containing malate as a carbon source) [41] as described previously [35]. In case of the strains with introduced pRK415 plasmid, kanamycin and tetracycline were added to the medium to the final concentrations of 25 mg/l and 2 mg/l, respectively. Chromatophores were prepared by sonication with high output power to yield smaller vesicles (average diameter of ≈30 nm) as in [34]. French-press treatment was used instead of sonication for preparation of chromatophores used in experiments presented in Figs. 1, 7 and 8. In the latter preparations the vesicle size was larger (~60 nm) and each vesicle presumably contained several ATP synthase molecules. Bacteriochlorophyll concentration was determined in acetone-methanol extract at 772 nm according to [42].

Introduction of the γMet23Lys mutation

Plasmid pRCAT1 was constructed from the plasmid pRCA50 (carrying the *Rb. capsulatus* F_1 operon inserted in pTZ18R [43]) by cutting the latter with *EcoRI* and ligating the 7.6 kb fragments with T4 DNA ligase.

The mutation was introduced into the pRCAT1 plasmid by using the QuickChange Site-Directed Mutagenesis Kit (Stratagene), using the following oligonucleotides for the PCR: 5'-CAAGATCACGAAAGCGAAGCAGATGGTCGCGG-3' and 5'-GTTCTAGTGCTTTCGCTTCGTCTACCAGCGCC-3'. Successful introduction was confirmed by restriction analysis with *HpyCH4V* restriction endonuclease, which produced a 1600 b.p. fragment in the mutated plasmid instead of ~900 b.p., ~700 b.p., and smaller fragments in the pRCAT1. The mutated F_1 operon was then cloned into the broad-host-range plasmid pRK415 [44] carrying the tetracycline resistance, as described previously [43]. The new plasmid was named pRCA51.23K and was subsequently introduced into *Rb. capsulatus* B100 strain by triparental conjugation [45] as modified in [43]. By this procedure, the wild-type chromosomal copy of the F_1-operon was deleted, and a kanamycin resistance cassette was introduced in its place by GTA (Gene Transfer Agent) transfer [46] and simultaneously the pRCA51.23K was introduced. A pseudo-wild-type strain was constructed in parallel by the same procedure, which harboured the plasmid pRCA51 (carrying the wild-type F_1 operon) and a kanamycin-resistance cassette instead of the chromosomal F_1 operon; this strain was used as a wild-type F_OF_1 control throughout the work. No major differences in the F_OF_1 properties between this pseudo-wild-type p51 strain and the B 100 wild-type without plasmids were observed.

Flash-spectrophotometric measurements

Chromatophores were suspended in the standard medium that contained 20 mM glycylglycine, 20 mM Na_2HPO_4, 100 mM potassium chloride or acetate, 5 mM magnesium chloride or acetate, 2 mM $K_4[Fe(CN)_6]$, 5 μM 1,1'-dimethylferrocene,

and 200 μM ADP. 2 mM KCN was present to ensure that no $\Delta\tilde{\mu}_H^+$ was generated in the darkness by cytochrome *c* oxidase; pH was 7.9. The final concentration of bacteriochlorophyll in the cuvette was 10-15 μM. Measurements were done at room temperature.

The kinetic flash-spectrophotometer used to monitor the flash-induced absorption changes was described previously [47]. Flash-induced changes in $\Delta\psi$ were monitored via electrochromic absorption band shift of carotenoid pigments at 522 nm (see [34] and references therein). The electrochromic absorption band shift was calibrated in millivolts of $\Delta\psi$ by imposing a K^+ diffusion potential in the presence of valinomycin as in [34]. According to the calibration, a single saturating actinic flash (10 μs full width at half-maximum) generated ≈70 mV of $\Delta\psi$. This value was lower than the corresponding flash-induced $\Delta\psi$ in the B10 *Rb. capsulatus* strain reported earlier [34] due to the higher ratio of bacteriochlorophyll to the photosynthetic centers (≈60:1 and ≈100:1 in B10 and B100, respectively).

Eight single traces recorded in the same sample were averaged to increase the signal to noise ratio. During the averaging, the time interval between the flashes was 12 s; it was long enough for the electrochromic signal to relax to its pre-flash background level. Monitoring light was cut off between the flashes to avoid additional excitation of the sample and $\Delta\tilde{\mu}_H^+$ generation. Three flashes at 12 s interval were given to each sample before measurements to avoid any effects of the longer than 12s incubations in darkness.

Changes of the pH inside the chromatophores were monitored by amphiphilic pH indicator neutral red [48] at 545 nm as in [34], but no bovine serum albumin was present (changes in pH of the bulk phase were effectively abolished by the pH buffers present). Nigericin, an electroneutral K^+/H^+ exchanger, was added to 1 μM to quench the flash-induced pH changes [25;49].

Measurements of ATP hydrolysis

When ATP hydrolysis was measured with the colorimetric pH indicator Phenol Red, chromatophores (10μM Bchl) were suspended in 0.5mM Tricine, 1mM $MgCl_2$, 25mM KCl, 0.2mM succinate, 100μM Phenol Red, pH 8.0. The reaction temperature was 25°C. The cuvette was illuminated from above by a light guide coming from a 250W quartz-tungsten halogen lamp, filtered by a colored glass long-pass filter with a cut-on wavelength of 780 nm. The pH changes of the suspension were followed as a function of time by the absorbance changes at 625-587 nm, and were calibrated after about 300 s of reaction by 3-fold addition of 25μM HCl. The overall pH change of the suspension at the end of the measurements was never higher than 0.3 units. The changes of proton concentration were transformed to changes of ATP concentration as described [50].

Measurements of ATP-driven proton pumping

ACMA fluorescence quenching assays were carried out in a Jasco FP 500 spectrofluorometer (wavelength 412 and 482 nm for excitation and emission respectively) at 25°C. Chromatophores were suspended to 10 μM bacteriochlorophyll in the following buffer: 0.5 mM Tricine, 50 mM KCl, 2 mM $MgCl_2$,1 mM NaPi, 0.2 mM succinic acid, NaOH to pH 8.0, an actinic effect of the excitation beam was eliminated by adding as inhibitor of the electron transport chain antimycin (5 μM) and by attenuating the excitation light by a 0.6 density filter (Ealing nr. 35-5818); ACMA was added to 0.7 μM. Prior to each measurement, the sample pH was adjusted to 8.0 with NaOH. Final ATP concentration was 600 μM. Measurements were done at room temperature.

Measurements of ATP synthesis.

The light-driven steady state ATP synthesis rate, as reported in Table 1 was measured at 25°C in the following buffer: 5 mM Tricine/NaOH, pH 8.0, 25 mM KCl, 1 mM $MgCl_2$, 2 mM Pi, 0.5 mM succinic acid, 10μM Bchl. The chromatophores suspension was illuminated from one side by a 250 Watts Xenon lamp and from the opposite side by a 150 Watts slide projector. The reaction was started by addition of 200 μM ADP. After stopping the reaction at various times with 6% trichloracetic acid, the ATP concentration in each sample was measured in a luminometer (LKB 1250) with the ATP-Monitoring Kit (Labsystems). The small amount of ATP synthesized in the dark (due to the adenylate kinase reaction) was subtracted. The amount of synthesized ATP was evaluated by adding 50-100 nM ATP.

ATP synthesis in response to the actinic flashes was measured as in [47]. Measuring medium was the same as in the flash-spectrophotometric experiments, but 0.2 mM luciferin and 5-15 U/ml luciferase were present. For the measurements of the flash-induced ATP synthesis and activation we used the same Xenon arc flash as for the flash-spectrophotometric experiments. The photomultiplier (Thorn EMI 9256B, UK) was shielded against actinic light by a stack of 3 blue filters (BG 39 Schott, Mainz, Germany). Measurements were done at room temperature.

The luciferin-luciferase system was calibrated in each sample by addition of freshly prepared ATP solution. The calibration was linear in the range of 0 to 5 μM final ATP concentration. Slight decrease in the sensitivity (which became more pronounced upon increase in the ATP concentration) during the measurements was taken into account by repetitive calibrations during and after each experiment. In the presence of ADP (without any ATP added) a minor ATP synthesis (up to 10 fM/s per mM BChl) insensitive to F_OF_1 inhibitors was observed, probably due to adenylate kinase activity of chromatophores. The latter activity resulted in increase of ATP concentration to 400-700 nM.

Results

Activation of ATP hydrolysis by continuous illumination

It was demonstrated previously that $\Delta\tilde{\mu}_{H}^{+}$ activates ATP hydrolysis in *Rb. capsulatus* chromatophores [28]. The increase in the ATPase activity in response to $\Delta\tilde{\mu}_{H}^{+}$ is best observed when the membrane is first energized, and then uncoupled. Under such conditions the enzyme stays activated for some time, while the back-pressure of $\Delta\tilde{\mu}_{H}^{+}$ is relieved and does not limit the rate of ATP hydrolysis. This behavior was reproduced in the chromatophores with the wild-type F_OF_1 used in this work (Fig. 1, trace a). After 30 s of illumination, $\Delta\tilde{\mu}_{H}^{+}$ generated by photosynthetic proteins was dissipated by switching off the light and by simultaneous addition of the uncouplers (nigericin and valinomycin). At this point a high rate of ATP hydrolysis was observed, which then slowly decayed. Oligomycin, a specific inhibitor that binds to F_O [51;52] and blocks the proton translocation [47] was used to confirm that this ATPase activity was coupled to proton transport through F_O (Panel A, trace b, d in Fig. 1). To calculate the rate of the coupled ATP hydrolysis, the traces obtained with oligomycin were subtracted from the traces recorded without the inhibitor (panel B in Fig. 1). After subtraction of the oligomycin trace, the initial rate of hydrolysis amounted to 134 mM ATP × M^{-1} BChl × s^{-1} (Panel B, trace a-b), and it decayed to the half after 28s. The γMet23Lys mutant similarly showed a high initial rate of hydrolysis (88 mM ATP × M^{-1} BChl × s^{-1}, Panel B, trace c-d), but the decay rate was markedly higher (half-life time 5s). These data are summarized in Table 1, together with the ATP hydrolysis rates measured in the dark without pre-illumination and with ATP synthesis rates measured under continuous light as described in the Materials and Methods. The values of the rates were obtained after best fitting the original data points (see Fig. 1B). The transient high rate of hydrolysis observed in the M23K mutant, although decaying very rapidly was consistently reproduced in different preparations. This

observation suggests that the mutated ATPase can indeed hydrolyze ATP efficiently, although the lifetime of its light-activated state is very short. This conclusion has been supported by further observations (described in Fig. 7, see below). It is also interesting to note that while the activated wild-type hydrolysis rate was 10-fold higher than the non-activated rate, the mutant rate was activated by a factor of 22 (see Table 1).

Effect of γMet23Lys mutation on the flash-induced proton transport through F_OF_1.

The results described above (Fig. 1 and Table 1) indicated that γMet23Lys F_OF_1 efficiently catalyzed ATP synthesis; the ATPase activity of the mutant enzyme was sensitive to F_O-inhibitor oligomycin and was stimulated by $\Delta\tilde{\mu}_H^+$. These findings imply that the coupling between F_O and F_1 was not lost, so we decided to investigate the proton translocation in the γMet23Lys mutant under ATP synthesis conditions using short flash of light for membrane energization. The excitation of *Rb. capsulatus* chromatophores by a single saturating actinic flash results in fast generation of $\Delta\tilde{\mu}_H^+$ across the chromatophore membrane (see [47] and the references therein for a detailed description of the flash-induced generation of the $\Delta\tilde{\mu}_H^+$ in *Rb. capsulatus* chromatophores).

This voltage generation can be monitored by electrochromic carotenoid absorption band shift at 522 nm, as shown in Fig. 2 and as described in Experimental Procedures. Absorbance changes at this wavelength are proportional to the changes in $\Delta\psi$ (see [34] and references therein), which in turn are proportional to the net charge transfer across the membrane.

The biphasic rise of the $\Delta\psi$ is followed by decay due to various ion fluxes including proton transport through the F_OF_1. The component of $\Delta\psi$ decay reflecting the proton escape from chromatophore vesicles can be obtained by recording traces with and without specific inhibitors and by calculating the respective ±inhibitor difference trace. To

determine the coupled proton transport we have used efrapeptin, a peptide antibiotic that binds inside F_1 between subunit γ and $\alpha_3\beta_3$ hexamer [53;54], whereas oligomycin has been used to estimate the total (coupled and uncoupled) proton transport. It was shown previously that the efrapeptin-sensitive component of $\Delta\psi$ decay correlates with proton uptake from the chromatophore interior and proton release into the bulk medium [34;55;56]. It was also shown that the extent of this $\Delta\psi$ decay component quantitatively correlates with ATP synthesis [47]. Thus, for the sake of simplicity below we refer to the ±efrapeptin traces as to "coupled proton transport".

Figure 2 illustrates the flash-induced $\Delta\psi$ changes and the coupled proton transport in chromatophores with wild-type F_OF_1 and with the γMet23Lys mutant enzyme. In correspondence with the results obtained previously [47], in chromatophores with the wild-type F_OF_1 a single flash in the presence of ADP and phosphate led to coupled proton transport of considerable extent (Fig. 2A). The data in Fig. 2 indicate that its maximal extent in the wild-type chromatophores was ≈ 15% of the total flash-induced charge transfer (compare traces +*Efrapeptin* and ±*Efrapeptin*). In contrast to the wild-type chromatophores, there was no detectable coupled proton transport under the same conditions in case of γMet23Lys mutant (Fig. 2B). Oligomycin also had no effect in γMet23Lys, ruling out insensitivity to efrapeptin as a possible effect of the γMet23Lys mutation (not documented).

When ATP was present at the final concentration of 2 mM, the coupled proton transport increased both in the wild-type and in the mutant (Fig. 2, panels C and D). The relative increase induced by ATP was much smaller in the wild-type sample. It should be noted that as the chromatophores had on average less than one active ATP synthase per vesicle, changes in the extent of the coupled proton transport reflected changes in the fraction of

active enzyme [34;35]. So the increase observed was likely due to activation rather than to change in the turnover rate of active enzyme.

To further characterize the effect, we investigated the dependence of the extent of the flash-induced coupled proton transport on ATP concentration (Fig. 3). It should be noted that even when no ATP was added to the sample, there was still some (≈ 0.5 μM) ATP present due to the contamination in ADP and to the adenylate kinase activity of chromatophores. Elimination of this residual ATP by glucose and hexokinase further diminished the extent of the flash-induced coupled proton transport in the wild-type enzyme (not documented).

A marked increase in the relative extent of the coupled proton transport with increase in ATP concentration was clear both in the wild-type and in the mutant γMet23Lys enzyme (Fig.3). In contrast to ATP, 1 mM AMP-PNP (a non-hydrolysable ATP analogue) failed to increase the extent of the coupled proton transport, indicating that not mere ATP binding, but ATP hydrolysis was necessary for the effect observed.

The results obtained were in apparent contradiction with thermodynamic considerations: increase in the concentration of the reaction product (ATP) was supposed to suppress rather than stimulate the reaction. However, it was in good agreement with the proposed above facilitated inactivation of the γMet23Lys mutant enzyme. We found probable that the $\Delta\tilde{\mu}_H^+$ generated by ATP hydrolysis during the dark adaptation time between the flashes could hinder this inactivation.

To validate this hypothesis we increased in the wild-type the dark adaptation time between the flashes during the trace averaging to provide more time for deactivation. The data presented in Fig. 4 indicate that the extent of the flash-induced coupled proton transport

declined to zero upon the increase of the time interval between the flashes. The time constant of deactivation was ≈ 10 s and was significantly higher than the time constant of $\Delta\psi$ decay (< 3 s). This observation suggested that the difference between the wild-type and the γMet23Lys F_OF_1 was merely in accelerated inactivation of ATP hydrolysis in the mutant, where no flash-induced coupled proton transport was detected even at shortest interval (12 s) between the flashes.

Activation of ATP hydrolysis at low ATP/ADP ratio.

To further clarify the role of γMet23Lys mutation in the deactivation of F_OF_1, and to investigate the $\Delta\tilde{\mu}_H^+$-activation of ATP hydrolysis by short flashes of light, we measured the flash-induced ATP synthesis and the subsequent ATP hydrolysis. A series of 1-20 flashes at 60 ms interval were given, and the concomitant ATP synthesis/hydrolysis were monitored by luciferin-luciferase system. The concentration of ATP before the actinic flashes was ≈ 1 μM; ADP concentration was 200 μM. As can be seen in Fig. 5 (panels A and C), in the wild-type chromatophores the rate of ATP hydrolysis, while negligible after one flash, increased markedly with the increase in the number of flashes. In contrast, in the γMet23Lys mutant (panels B and D) even a series of 20 flashes did not activate ATP hydrolysis, although considerable flash-induced ATP synthesis was observed.

The yield of ATP synthesized per flash in 20-flash series was similar in the wild-type and in the γMet23Lys mutant: 0.173 ± 0.035 mmol ATP × mol^{-1} BChl per flash for the wild-type and 0.206 ± 0.066 mmol ATP × mol^{-1} BChl per flash for the γMet23Lys mutant. This result indicated that the lower coupled proton transport observed in γMet23Lys mutant chromatophores was not due to lower expression level of the enzyme.

The role of $\Delta\psi$ and ΔpH in the activation of ATP hydrolysis.

To assess the individual role of the electrical and the chemical components of $\Delta\tilde{\mu}_{H}^{+}$ in the activation of *Rb. capsulatus* F_OF_1 under the experimental conditions used, valinomycin and nigericin were applied to selectively quench $\Delta\psi$ or ΔpH, respectively. As a measure of the activation, the difference between the rate of ATP hydrolysis before and after the flash series was taken. As can be seen in panels A and B in Fig. 6, no major changes in the extent of activation of the wild-type enzyme occurred upon quenching of ΔpH by 1 μM nigericin. This result was in good correspondence with earlier study reporting a negligibly small value of ΔpH generated in *Rb. capsulatus* chromatophores after a single flash in the presence of a pH-buffer glycylglycine [49]. A control experiment with the amphiphilic pH indicator neutral red confirmed the latter data (not documented).

When the flash-induced $\Delta\psi$ was abolished by 1μM valinomycin, no detectable activation was observed even after a series of 50 flashes (Fig.6C). However, increase in the flash number to 100 or 200 resulted in considerable acceleration of the ATP hydrolysis. It should be noted that a small residual absorption change at 522 nm was observed in response to flash series even in the presence of valinomycin (5-15% of the signal recorded in the absence of valinomycin; not documented). Therefore it cannot be excluded that some residual $\Delta\psi$ (< 30 mV) was generated under such conditions.

Control experiments with amphiphilic pH indicator neutral red (Fig.6C, inset) revealed that with such a high number of flashes a substantial ΔpH was generated even in the presence of 20mM glycylglycine and 20mM phosphate. This result might indicate that ΔpH could also efficiently contribute to the activation of ATP hydrolysis in the wild-type enzyme. Once again, no activation was observed in the γMet23Lys mutant (although some small degree of activation after 200 flashes cannot be excluded).

The data presented in Fig. 6 show that a single flash was not enough to achieve full activation of ATP hydrolysis in the wild-type chromatophores, indicating a relatively high

$\Delta\tilde{\mu}_H^+$ threshold for activation. Measurements of electrochromic absorption changes induced by a series of flashes revealed that the maximal $\Delta\psi$ value under repetitive flash excitation at 16.7 Hz (60 ms interval) was ≈ 170 mV and that it was reached after 5-6 flashes. The higher extent of the F_OF_1 activation upon the increase in the flash number from 5 to 10 and 20 indicated that the activation was a slow process and required relatively long (i.e. hundreds of milliseconds) exposure to $\Delta\tilde{\mu}_H^+$.

The results presented in Fig. 6 indicated that the activation of ATP hydrolysis by $\Delta\tilde{\mu}_H^+$ could not be detected in the γMet23Lys mutant under all the experimental conditions used (20 mM phosphate, 200 μM ADP, 1 μM ATP, and flash induced activation).

Activation of the hydrolysis by an ATP regenerating system

The inhibition of the ATPase by MgADP is a well established phenomenon in all ATP synthases and also in F_1 (see [57] and the references therein). Auto- and photoactivation of the ATPase in *Rb. capsulatus* chromatophores [28] can be related to the release of inhibitory ADP, consistent with the direct demonstration of this mechanism in the chloroplast enzyme [24]. In line with this view the addition of the pyruvate kinase (PK) /phospho-enol pyruvate (PEP) ADP trap that strongly reduces free ADP in the assay medium induced a stimulation of the hydrolysis rate in wild-type chromatophores (Fig. 7). Additions of increasing amounts of PK, thereby producing a progressively smaller concentration of ADP during the reaction, progressively stimulated the hydrolysis rate to a maximum asymptotic value.

Similar behavior was apparent in chromatophores from γMet23Lys mutant, although the reaction rates were systematically lower at all PK concentrations tested. However, the difference in the ATP hydrolysis rate of the wild-type and mutant F_OF_1 measured in the absence of ATP regenerated system was approximately fourfold, but only ~1.5-fold in the

presence of the latter (Table 1; Fig. 7). This result indicated that inhibition by ADP was enhanced in the γMet23Lys mutant.

The ATP hydrolysis measured in the wild-type in the absence of PK in Fig. 7 was 17 ± 3 mmol ATP · mol Bchl $^{-1} \cdot s^{-1}$ (average of 3 determinations). The higher value relative to that reported in Table 1 was due to the presence of 1 mM P_i, which is known to slightly stimulate the ATP hydrolysis in *Rb. capsulatus* (see e.g. (58)). On the contrary, no effect of P_i could be detected in the activity of the mutant enzyme, which was 4 ± 1 mmol ATP · mol Bchl $^{-1} \cdot s^{-1}$ (average of 3 determinations).

In these measurements, a kinetically limiting PK concentration can be excluded since, even at the lowest concentration, its activity was in about 20-fold excess relative to the ATP hydrolysis activity.

ATP-driven proton pumping in the γMet23Lys mutant.

It was reported previously that in *E. coli* ATP synthase the introduction of the γMet23Lys mutation severely impairs the coupling efficiency and leads to a complete loss of ATP-driven proton pumping [40]. In contrast, earlier measurements from the same group reported only a partial decrease in the coupling efficiency, and detectable (although markedly reduced) ATP-driven proton pumping [58].

Our results suggested that in *Rb. capsulatus* the mutant was coupled, as deduced from the oligomycin sensitivity of ATP hydrolysis and high ATP synthesis rate. To directly address this issue, we measured the ATP driven proton pumping in the wild-type and γMet23Lys chromatophores by the ACMA assay. As can be seen in Fig. 8, the mutant enzyme was active in proton pumping, although the initial rate was lower than in the wild-type. In all

cases the ACMA quenching induced by ATP was completely reversed by 0.5 μM nigericin (Fig. 8).

This result confirmed that in *Rb. capsulatus* ATP synthase the γMet23Lys did not abolish the ATP driven proton pumping under the experimental conditions used.

In an attempt to improve the comparison between the coupling efficiency of the γMet23Lys and of the wild-type F_OF_1, the rate of ATP hydrolysis in the wild-type enzyme was inhibited with 125 nM efrapeptin to a level observed in the non-inhibited mutant sample. Under these conditions the pumping rate in the two strains was very similar, suggesting that the reduced rate observed in the mutant was caused by lower hydrolysis rate rather than by lower efficiency of coupling.

Discussion

The γMet23Lys mutation does not affect the coupling efficiency in Rb. capsulatus F_OF_1.

Previously the effects of γMet23Lys mutation were extensively studied in *E. coli* F_OF_1. The mutation was found to slightly reduce the ATPase activity [36;37] and to impair markedly (although not completely) the coupling between ATP hydrolysis and proton pumping [58]. Another study reported a complete loss of the coupling [40]. Surprisingly, in single molecule experiments the mutated F_1 was shown to generate the same torque during ATP-driven rotation, and the rotation speed was also indistinguishable from those of the wild-type F_1 [40].

Our results indicated that in *Rb. capsulatus* the γMet23Lys mutation altered the activity of F_OF_1 in several ways. The most obvious effect was a more than threefold decrease in the rate of non-activated ATP hydrolysis (Fig. 1, Table 1, Fig 7). In contrast, ATP synthesis activity was only moderately impaired (less than two-fold under steady-state conditions, see Table 1). The enzyme also performed considerable ATP-driven proton pumping and no

marked difference in the coupling efficiency was detected between the wild-type and the mutant enzyme (Fig. 8). Moreover, the data in Fig. 2 indicate that no proton transport took place through the mutant enzyme in the presence of ADP and phosphate (although some efrapeptin-sensitive transport was readily observed after addition of ATP). These results confirmed that the mutant enzyme was not intrinsically "leaky" to protons. Absence of leaks in the entire membrane was also previously documented for the *E. coli* γMet23Lys mutant, by examining the proton pumping induced by lactate respiration [58].

A comparison of the amino acid sequences of the two enzymes demonstrates that the γ subunits are very conserved between the two bacteria: 115 over 290 amino acids are identical and most non-identical residues have similar hydrophobicity and charge of the side chain. The homology is even stricter for the β subunits that exhibit 69% identity and 81% similarity. It is likely, therefore, that our results with *Rb capsulatus* can be compared with a good degree of confidence to those obtained with *E. coli* ATP synthase, although, in principle, a different behavior between the two bacterial species cannot be excluded.

It is also conceivable that the uncoupling effects observed in *E. coli* γMet23Lys mutant were caused not by mutation itself, but by the specific experimental conditions used in these studies. Recent work on *Rb. capsulatus* F_OF_1 showed that the presence of ADP and phosphate, and possibly $\Delta\tilde{\mu}_H^+$, is critically important for efficient coupling [59]. The contrast between a complete lack of ATP-driven proton pumping in *E. coli* γMet23Lys mutant reported in [40] and clearly detectable (although small) proton pumping reported earlier in the same strain [58] also suggests that experimental conditions, but not γMet23Lys mutation per se, caused uncoupling. As discussed below, ADP concentration variations might have especially strong influence on γMet23Lys F_OF_1 activity.

Effect of γMet23Lys mutation on the activation of ATP hydrolysis by $\Delta\tilde{\mu}_H^+$.

The results presented in Fig. 1 and Table 1 indicated that activation of ATP hydrolysis by $\Delta\tilde{\mu}_H^+$ was present both in the wild-type and in the mutant. The relative activation of the coupled ATP hydrolysis after illumination was even higher in the mutant (Table 1). However, the deactivation occurring after uncoupling was markedly faster in the γMet23Lys F_OF_1 (half-time ≈5 s versus ≈25 s in the wild-type; Fig. 1).

Flash-induced coupled proton transport though the mutant F_OF_1 also differed from that in the wild-type enzyme. The data in Fig. 2 indicated that there was no detectable transport in γMet23Lys chromatophores unless ATP was added to the sample. However, upon addition of ATP the extent of the transport through F_OF_1 increased. This effect was also observed in the wild-type chromatophores, but in the mutant the relative increase was much higher.

The drop in the extent of the coupled proton transport indicated a decrease in the fraction of vesicles having an active enzyme [35;47], presumably due to the deactivation of the F_OF_1. So the results in Fig. 2 and 3 suggested that ATP prevented the deactivation. Absence of such effect in case of AMP-PNP, a non-hydrolysable ATP analogue (Fig. 3, *dark closed squares*), indicated that not merely ATP binding, but hydrolysis was necessary.

A probable cause for the effect observed would be ATP-driven generation of $\Delta\tilde{\mu}_H^+$. In this case the second and subsequent flashes would have increased the $\Delta\tilde{\mu}_H^+$ not from zero, but from a relatively high level determined by the phosphate potential (protonmotive force of ≈ 100 mV could be expected under the experimental conditions used with 1 μM ATP present and H^+/ATP ratio of 3.3). According to the calibration with K^+ diffusion potential (data not shown), a single flash generated ≈ 70 mV $\Delta\psi$ both in the wild-type (strain p51) and in the γMet23Lys mutant chromatophores. This value was well below the thermodynamic threshold for ATP synthesis. However, if this 70 mV was a surplus to a 100 mV background that corresponded to the phosphate potential before the flash, a considerable ATP synthesis would be expected.

This hypothesis was in good agreement with the observed decrease of the flash-induced coupled proton transport upon increase in the time interval between the flashes (Fig. 4). The magnitude of the flash-induced $\Delta\tilde{\mu}_{H^+}$ was approximately the same irrespective of the dark adaptation time, but the flash-induced coupled proton transport declined to zero upon increase in the interval. This indicated that in the dark the enzyme gradually lost the ability to perform coupled proton transport.

The data in Fig. 2A (recorded with 12 s dark adaptation before actinic flash) demonstrated that flash-induced coupled proton transport occurred in the wild-type chromatophores with no ATP added (i.e. the concentration of ATP was below 1 μM). In the framework of the rationale above, this implied that a considerable fraction of the wild type F_OF_1 remained active 12 s after the actinic flash even in the absence of added ATP. On the contrary, in γMet23Lys chromatophores a negligibly small coupled proton transport indicated that the deactivation was markedly faster. This result correlated well with the steady-state ATP hydrolysis data that also confirmed a facilitated and accelerated deactivation of the mutant enzyme after the actinic illumination was turned off (Fig. 1).

The results presented in Fig. 5 demonstrated that in contrast to the wild-type, at low ATP concentration (and high ADP concentration) the activation of ATP hydrolysis could not be detected at all in the mutant. It was also evident that no increase in the ATP hydrolysis rate was observed in γMet23Lys even in the very first seconds after the actinic flashes. Since the mutant enzyme did show high flash-induced ATP synthesis, indicating a highly active state, the most likely conclusion is that the mutant ATP synthase was deactivated immediately upon decrease of $\Delta\tilde{\mu}_{H^+}$ below the thermodynamic threshold of ATP synthesis.

Taken together, our results demonstrate that γMet23Lys mutation accelerated the transition from active to inactivated state of F_OF_1, most evidently at high ADP/ATP ratios.

γMet23Lys mutation might stabilize the ADP-inhibited state of the enzyme.

The deactivation caused by tight binding of MgADP at one of the F_OF_1 catalytic sites is a well-established mechanism of the enzyme regulation [14-21]. It is also demonstrated that upon the energization of the membrane the tightly bound ADP is released from the F_1-portion [20;22-25], that presumably leads to the $\Delta\tilde{\mu}_H^+$ activation of the enzyme.

Recent experiments revealed that mechanic rotation of subunit γ by 40º in the hydrolysis direction can also relieve the ADP-inhibition in F_1 [60]. Preliminary data reported in the latter work indicated that rotation of subunit γ by 160º in the synthesis direction had the same effect. Without forced rotation the spontaneous re-activation from the ADP-inhibited state is induced by thermal rotational fluctuations of subunit γ, and is completely blocked if the angular position of subunit γ is fixed by external force [60].

It was suggested from the experiments on *E. coli* F_OF_1 that impaired activity in γMet23Lys mutant was due to electrostatic interaction of the γLys23 with the first glutamate in the βDELSEED fragment [39]. It is possible that such interaction hindered γ rotation and thereby stabilized the ADP-inhibited state of the enzyme.

Consistent with this suggestion, ADP removal by the PEP/PK ATP regenerating system caused significantly more pronounced increase in the ATPase activity of *Rb. capsulatus* γMet23Lys mutant F_OF_1 (8-fold vs 4-fold in the wild-type, Fig. 7). This directly demonstrates that the inhibitory effect of ADP was enhanced in the mutant. The prompt inactivation of ATP hydrolysis in γMet23Lys F_OF_1 after activation by $\Delta\tilde{\mu}_H^+$ (Fig. 1), and the nearly undetectable ATP hydrolysis after trains of flashes at low ATP/ADP ratio (Fig.6) corresponded well with this suggestion.

In view of these findings it should be noted that even small variations in ADP concentration might have a pronounced effect on the ATPase activity of γMet23Lys F_OF_1. Therefore, it might be misleading to compare measurements of proton pumping with ACMA fluorescence quenching done without ATP regenerating system with measurements of ATPase activity done with such system.

The interaction of γLys23 with the first glutamate in βDELSEED seems probable according to the high-resolution crystal structures of the F_1-portion: the distance between the side chains of these residues is 3.4-4.7 Å [2;61-65]. This conclusion has recently got support from the structural study of the bovine enzyme inhibited by ADP and azide and resolved at 1.95 Å: azide stabilizes the inhibitory ADP in the beta-DP site, preventing the release of the nucleotide and the binding of phosphate [66]. Since routinely all crystals were grown in the presence of azide (about 3 mM), all less resolved structures showing an ADP binding site also contained non-resolved inhibitory azide and corresponded therefore to the ADP-inhibited conformation. The β-subunit with the DELSEED located close to γ23 residue bears the high-affinity catalytic site occupied by tightly bound ADP. According to the data obtained on the single molecule level, the angular position of the γ-subunit in the ADP-inhibited state (presumably caught in the crystal structures mentioned) differs by 40º from the "ATP-waiting" state observed under low ATP concentration [67]. This implies that the electrostatic interaction between γLys23 and the first Glu in the βDELSEED fragment is sterically impossible in the "ATP-waiting" active state. It might be that in the γMet23Lys enzyme the lifetime of the active "ATP-waiting" state is reduced due to stabilization of the γ-subunit angular position that corresponds to the ADP-inhibited state.

The impaired $\Delta\tilde{\mu}_H^+$ activation in the γMet23Lys mutant might also be responsible for the discrepancy between the pronounced effects of the mutation in the biochemical experiments and the absence of any detectable effect on the ATP-driven torque generation in the single-molecule rotational assays done on *E. coli* F_1 [40]. As was mentioned above, ADP-inhibition blocks the rotation of subunit γ [67], and spontaneous re-activation is induced by stochastic rotational movement of the γ-subunit [60]. Therefore, stabilization of the ADP-inhibited state in the γMet23Lys mutant proposed here would result merely in more frequent and prolonged pauses of subunit γ rotation, but not in a major decrease of the torque generated or of the turnover rate. As long as the enzyme is not trapped in the ADP-inhibited state, it is expected to perform ATP hydrolysis with the efficacy close to that of the wild-type F_1.

It should be noted that an alternative explanation for the inhibitory effect of γMet23Lys mutation was proposed by Bandyopadhyay and Allison from the experiments on thermophilic *Bacillus* PS3 $\alpha_3\beta_3\gamma$ complex. It was suggested that not the electrostatic interactions, but disruption of a hydrophobic cluster located on subunit γ in the vicinity of the βDELSEED is responsible for impairment of catalysis [68]. However, since the mutation was shown to change the activation energy for ATP hydrolysis [37], we still favor the hypothesis of electrostatic interactions between γLys23 and the first glutamate of the βDELSEED.

General conclusions on the activation of ATP hydrolysis by $\Delta\tilde{\mu}_H^+$.

Our results indicated that subunit γ plays a key role in the activation of ATP hydrolysis by $\Delta\tilde{\mu}_H^+$. It seems likely that such activation of F_OF_1 is merely a transition from the ADP-

inhibited state into active state caused by expelling of the tightly-bound ADP from one of the catalytic sites. This ADP release is most probably induced by the $\Delta\tilde{\mu}_{H^+}$-driven rotation of subunit γ. Our data point out that in *Rb. capsulatus* this rotation could be driven either by pure $\Delta\psi$ or by ΔpH in the presence of $\Delta\psi < 30$ mV. Electrostatic interactions of the negatively charged βDELSEED fragment with γLys23 in the mutant might stabilize the ADP-inhibited state and hinder subunit γ rotation and therefore impair the activation of ATP hydrolysis by $\Delta\tilde{\mu}_{H^+}$.

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Abbreviations:

F_OF_1 – H^+ transporting F_OF_1-ATP synthase; $\Delta\tilde{\mu}_H^+$ - transmembrane difference of proton electrochemical potential; $\Delta\psi$ - transmembrane difference of electrical potential; BChl - bacteriochlorophyll; ACMA – 9-amino-6-chloro-2-methoxy-acrydine;

FIGURE LEGENDS

Fig.1

Activation of ATP hydrolysis by $\Delta\tilde{\mu}_H^+$ in chromatophores of *Rb. capsulatus* wild-type and γMet23Lys mutant.

Changes of ATP concentrations were monitored by Phenol Red absorption changes as described in Materials and Methods. The chromatophores suspension (10 μM Bchl) in the cuvette was illuminated for 30s. After 25s of illumination 1 mM ATP was added (first arrow) and after additional 5s (second arrow) the light was switched off and at the same time uncouplers (0.4 μM nigericin and valinomycin) were added. Traces have been corrected for dilution and for the small absorption change following the pH change due to ATP addition.

Panel A. Trace a - wild type chromatophores, no inhibitors; trace b - wild type chromatophores, 20 μg/ml oligomycin; trace c - γMet23Lys mutant chromatophores, no inhibitors; trace d - γMet23Lys mutant chromatophores, 20 μg/ml oligomycin. Each trace is an average of two measurements.

Panel B. To reveal the oligomycin-sensitive activity, the traces obtained in the presence of oligomycin have been subtracted from traces recorded without the inhibitor. Trace a-b: wild type; traces c-d: γMet23Lys mutant. The continuous lines are obtained by best fit of bi-exponential functions to the data. The initial rates were 134 and 88 mol $\mathrm{ATP} \cdot \mathrm{mol\ Bchl}^{-1} \cdot \mathrm{s}^{-1}$ for pseudo-wild-type and γMet23Lys mutant, respectively. The inset shows an enlarged view of the same data.

Fig. 2

Flash-induced electrochromic traces recorded at 522 nm in the wild-type and γMet23Lys mutant chromatophores.

Medium contained 20 mM glycylglycine, 20 mM Na_2HPO_4, 100 mM KCl, 5 mM $MgCl_2$, 2 mM KCN, 2 mM $K_4[Fe(CN)_6]$, 5 μM 1,1'-dimethylferrocene, and 200 μM ADP; pH was 7.9. In panels **C** and **D** 2 mM ATP was present. After recording the *Control* trace, efrapeptin was added to final concentration of 200 nM. *Efrapeptin* trace was recorded 3 minutes after the addition of the inhibitor. ±*Efrapeptin* trace was obtained by subtracting the *Control* trace from the *Efrapeptin* trace. Bacteriochlorophyll concentration was 15 μM. Actinic flashes are indicated by arrows. Each trace is an average of 8 individual traces recorded at 12 s interval in the same sample.

Fig. 3

Dependence of the extent of the flash-induced coupled proton transport through F_OF_1 on ATP concentration. Measuring medium was as in Fig. 2.

Open circles – wild-type (strain p51) chromatophores; *closed grey squares* – γMet23Lys chromatophores; *closed black squares* – γMet23Lys chromatophores, but AMP-PNP was added instead of ATP. The extent of the ±*Efrapeptin* difference trace (see Fig. 2) was divided by the extent of the flash-induced electrochromic response of the photosynthetic reaction centres; the value at 1 mM ATP was taken as unity. At least three experiments were made for each ATP concentration. Standard error is plotted as bars.

Fig. 4

Dependence of the extent of the flash-induced coupled proton transport in the wild-type *Rb. capsulatus* chromatophores on the interval between the flashes.

Measuring medium was as in Fig. 2. Traces were averaged at different time interval (12 - 200 s, depicted on the *x* axis). The relative extent of the ±*Efrapeptin* difference trace was taken as a measure of the coupled proton flow through F_OF_1; the extent of the trace averaged at 12 s interval was taken as unity.

Fig. 5

Flash-induced ATP synthesis and activation of ATP hydrolysis in chromatophores of *Rb. capsulatus* wild-type and γMet23Lys mutant.

Changes in ATP concentration were monitored by luciferin-luciferase as indicated in Materials and Methods. ATP concentration was ≈ 1 μM. Traces were corrected for the background linear shift present before the flash series. Note the different scale on *y*-axis.

Each trace was recorded after at least 2 minutes dark adaptation. Bacteriochlorophyll concentration was 18 μM in the wild-type sample and 15 μM in the γMet23Lys mutant

sample. A – wild-type, 1 – 5 flashes; B - γMet23Lys, 1 – 5 flashes; C - wild-type, 1 – 20 flashes; D - γMet23Lys, 1 – 20 flashes.

Fig. 6

Activation of the ATP hydrolysis by flash-induced $\Delta\tilde{\mu}_H^+$.

The activation was measured as absolute increase in the initial rate of ATP hydrolysis (measured by luciferin-luciferase as in Fig. 5) after the series of actinic flashes. At least three measurements were done for each flash series (standard error plotted on each column).

A. No uncouplers. B. 1 μM nigericin present. C. 1 μM valinomycin present.

The inset in panel C illustrates the generation of ΔpH during the 200-flash series and absence of ΔpH generation when 1 μM nigericin was present (1 μM valinomycin was present in both experiments). The ΔpH was monitored by neutral red at 545 nm as described in Materials and Methods. The flash series is indicated by black bar with arrows.

Fig. 7

Dependence of the ATPase activity in the wild-type and γMet23Lys mutant chromatophores on the concentration of pyruvate kinase.

Measuring medium contained: tricine 10mM, KCl 50mM, $MgCl_2$ 4mM, succinic acid 0.2mM, lactate dehydrogenase 25U/ml, KCN 2.5mM, PEP 2mM, NaP_i 1mM, Antimycin 5μM, NADH 0.15mM, ATP 0.6mM.

In the measurements with no pyruvate kinase ATPase activity was measured by phenol red assay in tricine 1mM, P_i 1mM, KCl 50mM, $MgCl_2$ 4mM, Succinic acid 0.2mM, phenol red 100μM.

In all experiments pH was 8.0. Chromatophores were added to final concentration of 10μM bacteriochlorophyll.

Fig. 8

ATP-driven proton pumping in the wild-type chromatophores, in the γMet23Lys chromatophores, and in the wild-type chromatophores partially inhibited by 125 nM efrapeptin (so that the ATPase activity matched that of the uninhibited mutant sample). Chromatophores were suspended to 20 μM Bchl in 1 mM Tricine, 50 mM KCl, 4 mM $MgCl_2$, 1 mM NaPi, 0.2 mM succinic acid, pH 8.0; ACMA was added to 0.75 μM. Additions of ATP (600 μM) and of nigericin (500nM) are indicated by arrows.