FEBS Letters 455 (1999) 36–40 FEBS 22302

Active/de-active state transition of the mitochondrial complex I as revealed by specific sulfhydryl group labeling

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Received 19 May 1999

Abstract The sensitivities of NADH oxidase and/or NADH-ubiquinone reductase activities of submitochondrial particles and purified complex I towards *N*-ethylmaleimide (NEM) and other SH-reagents were studied. Only thermally de-activated preparations [A.D. Vinogradov (1998) Biochim. Biophys. Acta 1364, 169–185] were inhibited by SH-reagents whereas the redox-pulsed, activated enzyme was resistant to the inhibitors. The pH profile of the pseudo-first order inhibition rate suggested a pK_a of about 10 for the de-activation-dependent, NEM-reactive sulfhydryl group. NADH-ubiquinone reductase of activated particles treated with an excess of NEM followed by removal of the inhibitor was still capable of slow reversible active/de-active transition. When active, NEM-treated particles were de-activated and further inhibited by *N*-fluorescein maleimide, specific incorporation of the fluorescence label into low molecular mass polypeptide was evident. Comparison of the specific fluorescence labeling of submitochondrial particles, crude and purified complex I showed that the active/de-active state-dependent SH-group is located in a 15 kDa polypeptide (most likely in the 15 kDa IP subunit of the iron-sulfur protein-containing fraction of complex I).

Key words: NADH-ubiquinone oxidoreductase; Complex I; Respiratory chain; Fluorescence labeling; Bovine heart mitochondrion

1. Introduction

Mammalian mitochondrial NADH-ubiquinone oxidoreductase (EC 1.6.5.3, complex I) is an extremely complex multiple redox component energy-transducing unit of the respiratory chain which is composed of more than 40 different polypeptides [1]. Little is known about the functions of the individual subunits. Five of them (75 kDa IP, 51 kDa FP, 24 kDa FP, 23 kDa TYKY and 20 kDa PSST) are generally assigned for possible iron-sulfur cluster location [2]. The 51 kDa FP subunit is believed to carry FMN and NADH/NAD^+ binding sites [1,3]; the 39 kDa subunit also contains a sequence that fits the nucleotide binding motif [1]. A protein (15 kDa) capable of ubiquinone binding has been isolated from the IP fraction obtained after resolution of complex I [4]. An acyl carrier protein has been detected as an intrinsic constituent of bovine heart [5] and *Neurospora crassa* [6] complex I. Thus specific functions have been ascribed to only eight out of more than 40 subunits.

The catalytic properties of mammalian complex I are also not trivial. The slow active/de-active state transition is a notable feature of the enzyme [7]. Some essential characteristics of this phenomenon are summarized below. (i) After exposure of the enzyme preparations (submitochondrial particles [8] or purified complex I [9]) to elevated temperature (30–37°C) the rotenone-sensitive NADH oxidation or energy-linked ubiquinol-NAD^+ reduction demonstrates a considerable lag phase in continuous assays. The only treatment which eliminates the lag phase is preincubation of thermally 'de-activated' preparations in the presence of NADH (or NADPH) under conditions where electron transfer from the substrate to ubiquinone (i.e. enzyme turnover) is permitted [8,9]. Neither ferricyanide reductase nor hexaammine ruthenium(III) reductase activities are affected by the thermally induced de-activation. (ii) The lag phase is strongly pH-dependent (increases at alkaline pH) and can be almost indefinitely prolonged by the presence of divalent cations whereas the final steady-state 'activated' rate of NADH oxidation is insensitive to Ca^{2+} or Mg^{2+} and only slightly pH-dependent [10]. (iii) An equilibrium between active and de-activated enzyme forms (greatly shifted to the latter) is reached in the absence of any ligands and the rate of equilibration is exceptionally temperature-dependent: it is negligible at ambient temperature (15–20°C) and becomes quite significant (half-time in minute scale) at 30–37°C [11]. (iv) Only thermally de-activated enzyme is irreversibly inhibited by SH-reagents such as (*N*-ethylmaleimide) NEM [10].

Th very high activation energy for the equilibration seems to suggest gross conformational rearrangement of the protein structure during active/de-active enzyme transition, at least in that part of the enzyme which is involved in rotenone-sensitive ubiquinone reduction. What particular subunit(s) is (are) involved in the dramatic change of the catalytic properties remains to be established. In this report we will show that a 15 kDa polypeptide in de-activated preparations of complex I is specifically labeled by the fluorescence analogue of NEM. Although the primary structure of this subunit and the precise position of the labeled amino acid residue remain to be established, the 15 kDa IP protein is suggested to be the most likely component responsible for (or involved in) the slow active/de-active enzyme transition.

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Abbreviations: NEM, *N*-ethylmaleimide; FM, *N*-fluorescein maleimide; FP, three-subunit flavo-iron-protein derived from complex I; IP, iron-sulfur-enriched protein fraction derived from complex I; SDS, sodium dodecyl sulfate; SMP, submitochondrial particles; BSA, bovine serum albumin; DTNB, dithiobis-nitrobenzoic acid; DTT, dithiothreitol; Q_1, 2,3-dimethoxy-5-methyl-6-isoprenyl-1,4-benzoquinone

PII: S0014-5793(99)00850-9

2. Materials and methods

Bovine heart submitochondrial particles (SMP) [8] and complex I [12] were prepared according to the published procedures.

2.1. Fluorescence labeling

The following procedure was worked out for specific labeling of active/de-active state-dependent sulfhydryl group(s). SMP (1 mg/ml) were suspended in 0.25 M sucrose, 1 mM EDTA, 50 mM Tris-Cl^- (SET buffer), pH 8.0. 0.5 mM NADPH was added and the mixture was incubated with vigorous stirring for 30 min at 20°C in an open vessel to activate complex I [13]. The suspension was cooled (0–4°C), the particles were precipitated (120 000 × *g*, 45 min), suspended (~40 mg/ml) in 0.25 M sucrose and stored in liquid nitrogen (A-SMP). A-SMP were suspended (10 mg/ml) in SET buffer, pH 9.0 and treated with 30 mM NEM at 15°C for 30 min. The reaction was stopped by 10-fold dilution of the suspension with SET buffer containing 5 mM cysteine (pH 7.5), and particles were sedimented (105 000 × *g*, 30 min). The pellets were washed with SET buffer containing no cysteine, and suspended (~50 mg/ml) in 0.25 mM sucrose (AN-SMP). AN-SMP suspended in SET buffer, pH 8.0 (10 mg/ml), were divided into two equal 1 ml parts. One part was incubated for 90 min at 30°C (DN-SMP); the other part was kept in a refrigerator (AN-SMP). Both samples were placed in a water bath (15°C), 0.5 mM FM was added, and incubation was continued in the dark for 12 min. The reaction was stopped by 10-fold dilution with SET buffer containing 5 mM cysteine, the particles were precipitated (105 000 × *g*, 30 min), washed with the same buffer, and each sample was suspended in 0.25 M sucrose (ANF*-SMP and DNF*-SMP, respectively). Both samples were further subjected to red-green split [14] and final preparations (ANF*-S_1 and DNF*-S_1) were suspended in 0.25 M sucrose.

Essentially the same procedure (NEM treatment and further FM treatment) was used for preparation of fluorescence-labeled samples of active and de-activated purified complex I. For easier reading the procedure described above is presented as a flow diagram in Fig. 3.

2.2. SDS electrophoresis

The samples of SMP and complex I treated as described were mixed with 9 volumes of cold acetone (10 min vigorous mixing to extract phospholipids), and protein was precipitated by 5 min centrifugation at 2000 × *g*. We found that phospholipid extraction significantly improves electrophoretic resolution of the polypeptide bands, especially in the low molecular mass region. The protein samples were dissolved in a mixture containing 0.06 M Tris-Cl^- (pH 7.0), 8 M urea, 2% SDS and 5% mercaptoethanol and subjected to electrophoresis according to Laemmli [15]. The details are described in the legend to Fig. 4.

2.3. Other methods

NADH oxidase activity was assayed spectrophotometrically as absorbance decrease at 340 nm at 36°C in a reaction mixture containing 0.25 M sucrose, 0.2 mM EDTA, 50 mM Tris-Cl^-, pH 8.0, 1 mg/ml BSA, 0.2 μg/ml gramicidin D, and 0.1 mM NADH. NADH-ubiquinone reductase activity was assayed in the same mixture containing 1 μg/ml antimycin A and 80 μM Q_1. Protein content was determined with biuret reagent.

NADH, NADPH, BSA, EDTA, Q_1, D,L-cysteine, NEM, DTNB, glycine, gramicidin D, and antimycin A were from Sigma (USA). Tris (base), SDS, and DTT were from Serva (Germany). Fluorescein maleimide was from Molecular Probes (USA). Other chemicals were of the highest quality commercially available.

3. Results

The main purpose of this work was to identify a component of complex I which is responsible for (or involved in) the slow transformation of the enzyme activity. The selective sensitivity of the de-activated form of complex I [10] to SH-reagents and the extreme temperature dependence of the transition were used as the guidance for specific fluorescence labeling of the enzyme subunits. Fig. 1 demonstrates a dramatic difference in the sensitivity of enzymatic activities of the active and de-activated preparations towards NEM. Only a small fraction of NADH oxidase was inhibited when NADPH-treated (active) enzyme was incubated with a large molar excess of NEM at 15°C. In contrast, rapid and complete inactivation was observed when the same preparation of SMP was subjected to NEM treatment at 15°C after preincubation at 30°C (de-activated enzyme). When activated particles treated with NEM (AN-SMP) were separated, they retained insensitivity to the sulfhydryl group reagent and became rapidly inhibited by NEM or its fluorescence analogue after thermally induced de-activation (DN-SMP, Fig. 1B).

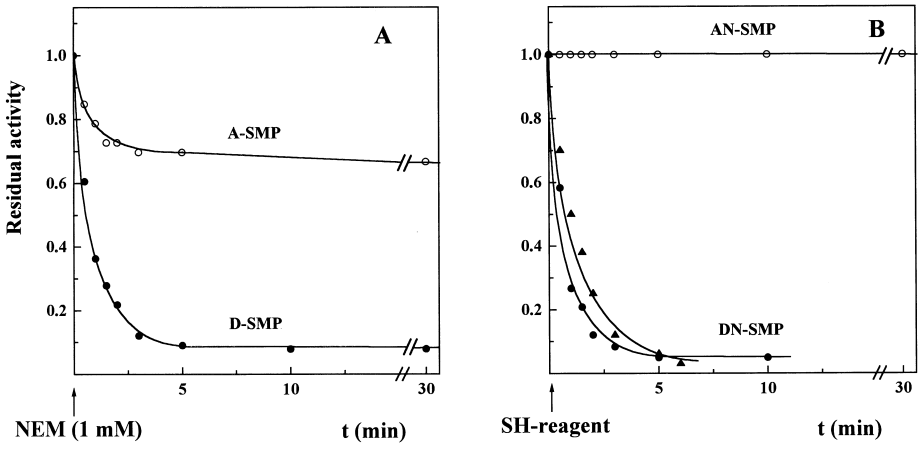

Fig. 1. Inhibition of NADH oxidation by SH-reagents in different preparations of SMP. A: Activated (A-SMP) and de-activated (D-SMP, A-SMP preincubated for 1.5 h at 30°C) particles (10 mg/ml) were kept at 15°C in a mixture containing 0.25 mM sucrose, 1 mM potassium EDTA and 50 mM Tris-Cl^- (SET buffer), pH 8.0. 1 mM NEM was added at time zero. The NADH oxidase activity was assayed. The original activity corresponds to 1.7 μmol of NADH oxidized per min per mg protein. A prominent lag phase in NADH oxidase of de-activated particles was observed [7] whereas simple zero-order NADH oxidation was seen for activated preparations. The final constant rates of NADH oxidation are plotted on the ordinate. B: A-SMP (10 mg/ml) were incubated at 15°C in the presence of 1 mM NEM as shown in A for 30 min. The mixture was diluted in SET buffer containing 5 mM cysteine, particles were sedimented by centrifugation, washed with the same buffer containing no cysteine and suspended in 0.25 mM sucrose. The sample was divided into two parts; one part was de-activated (incubation for 70 min at 30°C, DN-SMP); the other part was kept in ice (AN-SMP). Both samples were then subjected to the inhibitors at 15°C as described in A. Arbitrary unit of activity corresponds to 1.1 μmol of NADH oxidized per min per mg protein. 1 mM NEM (●) or 1 mM FM (▲) was added as SH-reagent.

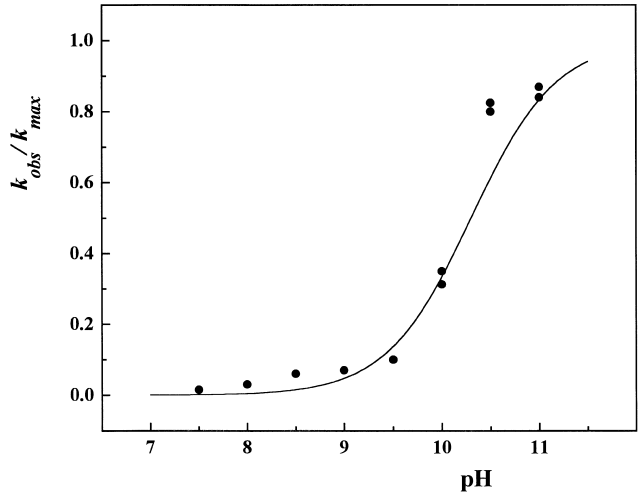

Fig. 2. pH dependence of inhibition of NADH-ubiquinone reductase by NEM in de-activated submitochondrial particles. SMP (0.5 mg/ml) de-activated at 30°C as described in Fig. 1 were incubated in SET buffer (pH is indicated) in the presence of 25 μM NEM at 20°C. The time course of the residual NADH-Q_1 reductase activity was followed as described in Fig. 1 and the pseudo-first-order inhibition rate constants (k_{obs}) were calculated from the semilogarithmic linear plots (log of the residual activity versus incubation time) for each pH. The continuous line corresponds to the theoretical curve for alkylation rate of a single sulfhydryl group with $pK_a = 10.2$ if k_{max} (pseudo-first-order rate constant) is assumed to be 2 min^{-1}.

The pH dependence of the pseudo-first-order rate constant for inhibition of de-activated preparations by NEM is shown in Fig. 2. Since de-protonated SH-groups are known to react with alkylating NEM much more rapidly than protonated residues [16], the pH profile corresponds to an apparent pK_a of the group. The value of 10.2 is considerably higher than that expected for dissociation of an 'average' sulfhydryl in aqueous environment. Because of the unusual pH profile shown in Fig. 2 and because NEM is able to react with amino groups at alkaline pH it was desirable to confirm that the inhibition was, indeed, due to SH-group reactivity. Qualitatively the same results as those shown in Fig. 1 were obtained when the following reagents were used as the inhibitors: DTNB, iodoacetamide, monobromobimane (results not shown). The inhibition of de-activated enzyme by DTNB was completely reversed after treatment with an excess of DTT. When the total amount of 'SH-groups' in SMP was determined with Ellman's reagent (pH = 8.0, no detergents added), a value of 16 ± 1 nmol/mg protein was obtained. Assuming a complex I content in SMP of 0.1 nmol/mg protein [11], the relative amount of the enzyme NEM-reactive groups could be approximated as less than 1% of the total. Thus, the possibility of tracing a specific NEM-sensitive group during inhibition of the enzyme activity seemed to be difficult if not impossible. However, the marked resistance of NADH oxidase to NEM at 15°C found for 'active' preparations could be utilized for a significant decrease of 'background' SH-group content. The conditions for alkylation of those background SH-groups were found based on the rate constant for NEM-induced inhibition, its pH dependence and the marked resistance of activated enzyme to the inhibitor at 15°C (see Fig. 1). On the assumption that all sulfhydryl groups in SMP are equally reactive with NEM except for those which become reactive only after thermally induced de-activation, the procedure depicted as a flow diagram in Fig. 3 was worked out. The key steps consisted of treatment of activated particles with a large molar excess of NEM, removal of unreacted reagent, de-activation and chase incubation of NEM-treated particles with fluorescence derivative of NEM (FM). The fluorescence-labeled particles were then resolved (red-green split usually employed as a first step for solubilization of complex I) and solubilized fractions (S_1) were subjected to electrophoresis. Essentially the same procedure was applied for preparation of active and de-activated purified complex I. Electrophoretic patterns as visualized by the protein bands staining and fluorescence labeling are shown in Fig. 4. Although a meaningful identification of the numerous bands seen after electrophoretic resolution of submitochondrial particles was, as expected, difficult, a clear difference in fluorescence labeling was evident: de-activated particles showed a much more intense single fluorescence band in the low molecular mass region (indicated by arrow). Other bands which were detected by fluorescence and all bands revealed by protein staining were identical. The same protein bands were recovered in the solubilized fractions (S_1) and the difference in fluorescence intensities for the samples obtained from active and de-activated particles was associated with the same low molecular mass band. Much better resolution was obtained when active

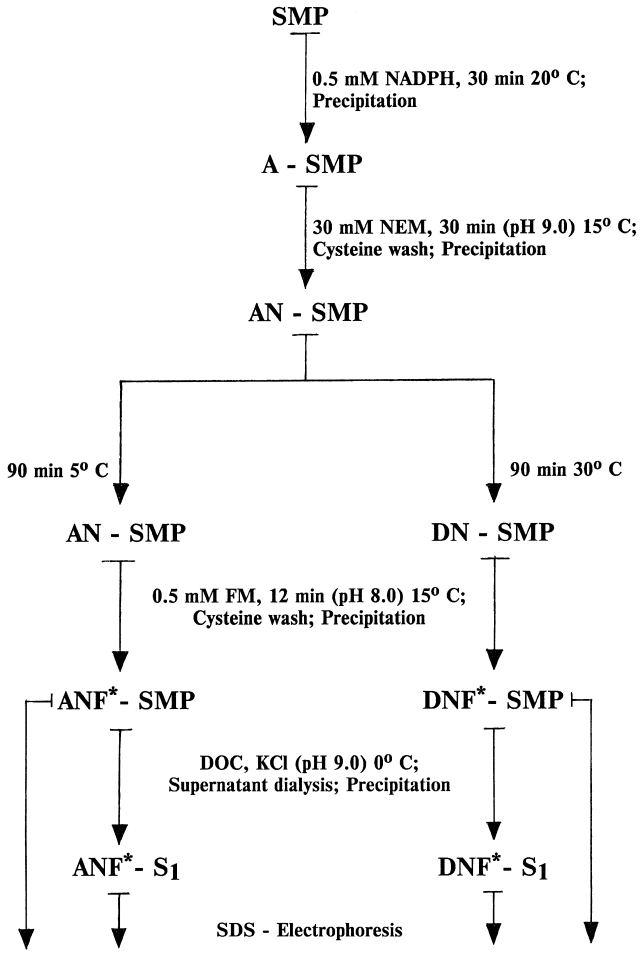

Fig. 3. Flow diagram showing a procedure for preparation of the samples to be analyzed by SDS-electrophoresis. The details are described in Section 2.

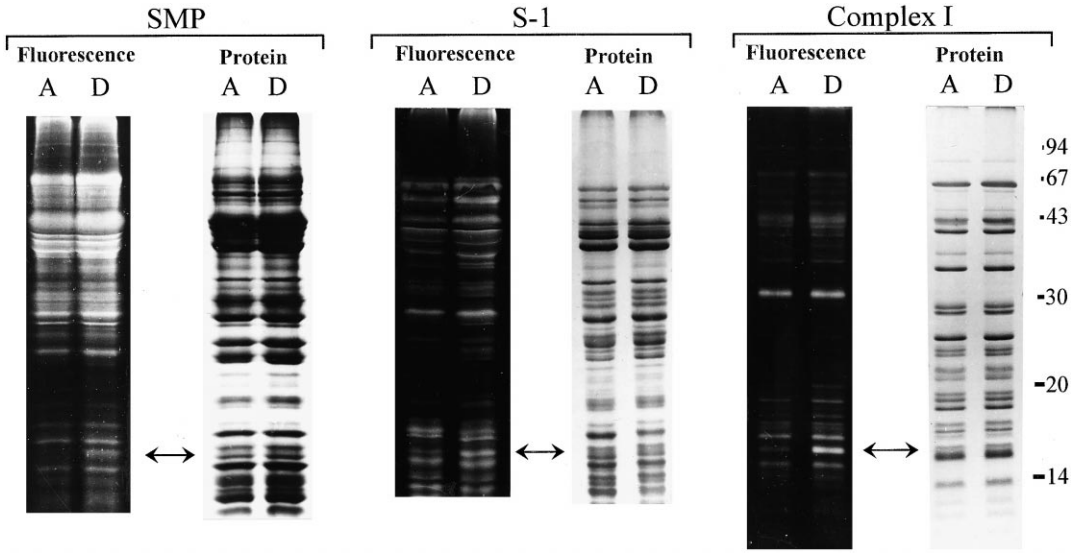

Fig. 4. Identification of the de-activation-dependent labeled subunit in the preparations of complex I at different degrees of resolution. The samples of SMP and S-1 were prepared as depicted in Fig. 3. Equal volumes of active (ANF*-SMP, ANF*-S_1 and complex I) and de-activated (DNF*-SMP, DNF*-S_1 and complex I) preparations were applied on each track (about 100, 60 and 30 μg of protein for SMP, S-1 and complex I, respectively). 12.5% (SMP and S-1) and 15% (complex I) acrylamide gels were used. After electrophoresis was completed the gels were placed on a UV transilluminator and photographed through the optical filter (λ_{max} 510 nm). The positions of fluorescence bands were marked and gels were further stained (Coomassie R-250) and destained. The destained gels were photographed on a white light transilluminator. Because of swelling the actual size of the gels after the staining/destaining procedure was slightly larger than those documented in UV light. Proper corrections were made when photographs were printed for exact positioning of the bands. The molecular mass scale (on the right) corresponds only to purified complex I preparations. Tracks A and D correspond to active and de-activated preparations, respectively (see Fig. 3).

and de-activated preparations of purified complex I were subjected by SDS-electrophoresis after fluorescence labeling. Again, the subunit composition was exactly the same in active and de-activated preparations and the only subunit with an apparent molecular mass of 15 kDa showed a strong difference in fluorescence intensity. Thus, we conclude that inhibition of de-activated NADH-quinone reductase by the fluorescence derivative of NEM (see Fig. 1) is accompanied by specific incorporation of the inhibitor into the 15 kDa subunit of complex I.

4. Discussion

The data presented here extend the original observations on the activation/de-activation phenomenon previously characterized solely by kinetic approaches [17]. The location of the de-activation-dependent, SH-reagent-sensitive group is now unambiguously established in the 15 kDa subunit of complex I. At least two polypeptides with an apparent molecular mass of 15 kDa are present in complex I, these are 15 IP and B 15 according to the nomenclature of Walker et al. [18]. The final assignment of the NEM- (and/or other SH-reagent-) reactive subunit should await the primary amino acid sequence determination of fluorescence-labeled polypeptide and identification of particular labeled residue. The 15 IP subunit seems to be the most likely candidate because B 15 contains no cysteine residues, whereas 15 IP (PFFD N-terminal sequence) has four cysteine residues (C-32, C-42, C-55 and C-65) in its 105 amino acid mature protein sequence with no N-terminal import signal extension [18]. The hydropathy profile of 15 IP suggests that it contains no hydrophobic sequence fragments to be folded into membrane-spanning α-helices. On the other hand, it has been reported that the 15 kDa polypeptide of IP is reactive towards monospecific antibodies in inside-out submitochondrial particles and in mitoplasts [19]. The pK_a value of 10.2 for the specific NEM-reactive group (Fig. 2) suggest its location close to the negatively charged membrane surface. It is worth remembering that the catalytic turnover-dependent activation of complex I is strongly pH- and divalent cation-dependent [10]. We propose that protonation of the SH-group facilitates dislocation of cysteine residues from a hydrophilic environment (de-activated enzyme) into a hydrophobic intramembranous environment (active enzyme) where it becomes inaccessible for SH-reagents. The divalent cations decrease the activation rate by lowering the SH-group pK_a resulting from compensation of the membrane negative charge.

It has been claimed that the relative amount of the 15 IP subunit varies from one preparation of IP to another and that it demonstrates anomalous behavior when IP is further fractionated [20]. Since all preparations of complex I are heterogeneous in terms of the active/de-activated state of the enzyme [7], the anomalies in 15 IP distribution can be explained by its different conformation in either state. It is worth noting that the 15 kDa region of electrophoretically resolved complex I samples often (not always) appears as a triplet (see Coomassie-stained gels in Fig. 4 and also the electrophoretic patterns in [2]). Although three bands are clearly seen in the gel system of Laemmli when applied for separation of the bovine heart complex I subunits [18], only two of them (B 15 and 15 IP) were assigned in the suggested nomenclature. It cannot be excluded that the subunit we have identified by fluorescence labeling is a new constituent of complex I previously not considered an intrinsic enzyme component.

Acknowledgements: This work was supported by National Institute of Health (USA) Fogarty International Research Collaborative Grant R03 TW00140-01A2, by the Russian Foundation for Fundamental Research (Grant 96-04-48185) and by the Program of Advanced

Schools in Science (Grant 96-15-97822). We are grateful to Dr. V.G. Grivennikova for critical comments and help in preparation of the manuscript.

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