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Mechanism of Electron Transfer in Fusion Protein Cytochrome b_5–NADH-cytochrome b_5 Reductase

A. V. Yantsevich, A. A. Gilep, and S. A. Usanov*

Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, ul. Kuprevicha 5, 220141 Minsk, Belarus; fax: 375 (172) 63-7274; E-mail: usanov@iboch.bas-net.by

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Abstract—In the present work we summarize results on construction of expression plasmid, heterologous expression in *Escherichia coli*, isolation and purification, as well as physicochemical characterization of chimeric protein consisting of hydrophilic domain of cytochrome b_5 and truncated from the N-terminal sequence (Δ^{23}) form of NADH-cytochrome b_5 reductase. The kinetics and mechanism of electron transfer between NADH-cytochrome b_5 reductase and cytochrome b_5 in the frames of fusion protein consisting of cytochrome b_5 (94 amino acids) and truncated form of NADH-cytochrome b_5 reductase (277 amino acids) have been studied. It is shown that electron transfer takes place between redox partners belonging to two different molecules of the chimeric protein. Using computer modeling, we built the model of the tertiary structure of the fusion protein, which is in agreement with experimental data. By using Marcus theory of electron transfer in polar media, we demonstrate the inability of the hypothesis of electrostatic repulsions to explain the increase of electron transfer rate on increase of ion concentration in media due to elimination of the repulsion of similar charges. The real reason for the increase of the first order rate constant in some oxidation–reduction reactions between proteins, as shown in the present work, is a decrease of the media reorganization energy resulting in decrease of activation energy for oxidation–reduction reactions.

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NADH-cytochrome b_5 reductase and cytochrome b_5 are a widely distributed among eukaryotic organisms pair of redox partners that performs a set of very important functions [1, 2]. Both proteins are usually located in endoplasmic reticulum membranes and interact with membrane via their N- and C-terminal hydrophobic sequences, respectively [3, 4]. Besides endoplasmic reticulum membranes, a membrane-bound form of NADH-cytochrome b_5 reductase is also found in mitochondria and in nuclear and plasmatic membranes, where it participates in desaturation and elongation of fatty acids, hydroxylation reactions with participation of cytochrome P450, and cholesterol biosynthesis [5, 6]. Erythrocytes were found to contain a "soluble" form of NADH-cytochrome b_5 reductase, which contains a catalytic FAD-containing domain of full-length NADH-cytochrome b_5 reductase and is the product of alternative transcription and splicing of the same gene [7]. This form of NADH-cytochrome b_5 reductase together with cytochrome b_5 is responsible for reduction of methemoglobin in erythrocytes [8].

Cytochrome b_5 is a hemeprotein (16 kD) consisting of two functional domains—a hydrophilic heme-containing domain preserving all spectral properties of the full-length hemeprotein and a short hydrophobic C-terminal domain (~40 amino acids) responsible for the interaction of cytochrome b_5 with the microsomal membrane [1, 2]. Cytochrome b_5 plays an important role in such physiologically relevant reactions as desaturation of fatty acids [9], methemoglobin reduction in erythrocytes [8], hydroxylation of N-acetyl-neuraminic acid [10], as well as participating in different oxidation reactions mediated by cytochrome P450 [11].

Cytochrome b_5 forms specific complexes in which electron transfer takes place with cytochrome *c* [12] and their physiological partners—NADH-cytochrome b_5 reductase [13], NADPH-cytochrome P450 reductase [14], and various forms of cytochrome P450 [15-17].

The leading role in complex formation between cytochrome b_5 with NADH-cytochrome b_5 reductase is played by electrostatic interactions between negatively charged amino acid residues surrounding the heme of cytochrome b_5 and the positively charged residues of the

* To whom correspondence should be addressed.

redox electron partner [17]. There are at least two main clusters of negatively charged residues in cytochrome b_5. Residues Glu48, Glu49, and Asp65 appear to be involved in interaction with electron acceptors, in part with cytochrome *c*, while, located on the other side of the surface residues Glu42, Asp71, and Glu74, participate in complex formation with electron donor—NADH-cytochrome b_5 reductase [15]. Besides electrostatic interaction, mediated mainly by the catalytic hydrophilic domain, an important role is also played by hydrophobic interactions, primarily via the C-terminal hydrophobic domain of cytochrome b_5 [16, 18, 19].

NADH-cytochrome b_5 reductase and cytochrome b_5 are often involved in formation of more complex proteins. Analysis of sequenced genomes indicates that mammalian cells (human, mouse, rat) contain a gene coding a chimeric protein consisting of the hydrophilic domain of cytochrome b_5 and the hydrophilic domain of NADH-cytochrome b_5 reductase [20]. The human chimeric protein was recently expressed, and it was shown that in the presence of NADH it is able to generate a reactive oxygen form (superoxide anion) [21].

Proteins fused to the single polypeptide chain consisting of hemeproteins and flavoproteins—flavohemeproteins, are widely distributed in nature. Cytochrome P450BM3 belongs to this group; it is a natural fusion protein consisting of cytochrome P450 and FAD-,FMN-containing flavoprotein—NADPH-cytochrome P450 reductase—which is the most active catalyst in oxidation of fatty acids [22]. Different forms of NO-synthase also belong to the same group, that is, they are also fusion proteins between cytochrome P450 and NADPH-cytochrome P450 reductase, but in contrast to cytochrome P450BM3, they contain additional calmodulin- and tetrahydrobiopterin-binding sites [23]. The electron transfer from flavoprotein to hemeprotein in cytochrome P450BM3 is intramolecular, with participation of a thermodynamically unstable semiquinone radical of FMN [24], while in the case of NO-synthase electron transfer occurs from FMN of flavoprotein of one NO-synthase molecule to heme of cytochrome P450 of another molecule [25].

To understand the functional role of the chimeric protein NADH-cytochrome b_5 reductase–cytochrome b_5 and to study the mechanism of intra- or intermolecular electron transfer in electron transfer chains, in the present work we constructed, expressed in *E. coli* cells, and purified to apparent homogeneity an artificial chimeric protein consisting of cytochrome b_5 and NADH-cytochrome b_5 reductase. In the present work we present the results of kinetic analysis of the electron transfer reaction between redox partners fused into a single polypeptide chain by using for interpretation of experimental data Marcus electron transfer theory for solutions [26]. The results indicate that the increase of electron transfer rate for some oxidation–reduction reactions at increased ionic strength of the reaction medium is the result of decrease of the energy of media reorganization, which results in decrease of the activation energy of the oxidation–reduction reactions.

MATERIALS AND METHODS

Chemicals. In the present work we used: sodium cholate, 3-[(3-cholamidopropyl) dimethylammonium]-1-propansulfonate (Chaps), SDS, Coomassie R-250, albumin, NADPH (Sigma, USA); Emulgen 911 and Emulgen 913 (Kao Atlas, Japan); phenylmethylsulfonyl fluoride (PMSF), low-melting agarose, isopropyl-β-D-thiogalactopyranoside, dithiothreitol (BRL, USA); bacto-tryptone and yeast extract (Difco, USA).

Engineering of expression plasmid for chimeric protein NADH-cytochrome b_5 reductase–cytochrome b_5. The cDNA coding full-length rat (*Rattus norvegicus*) NADH-cytochrome b_5 reductase was amplified by PCR with specific primers. The primers were constructed based on nucleotide sequence of rat NADH-cytochrome b_5 reductase [27]. The 5′-primer (N-terminal primer) allows including in the N-terminal sequence restriction site *Sal* I, which is further used for cloning into expression vector. The 3′-antisense primer (C-terminal primer) introduces the *Xba* I restriction site. Amplification products were ligated into pGEM-T vector (Promega, USA). Correspondence of nucleotide sequences obtained to expected ones was confirmed by sequencing.

The *Sal* I-*Xba* I fragment of the pGEM-Nb5R vector was further cloned to pCWori+b5_ncpr vector instead of the fragment coding NADPH-cytochrome P450 reductase [28]. As a result, we constructed pCWori+b5_nb5r expression vector (Fig. 1, see color insert). The construct obtained was checked with restriction analysis and sequencing.

The gene coding fusion protein cytochrome b_5–NADH-cytochrome b_5 reductase contains 1131 bp that corresponds to 377 amino acid residues. Analysis of the sequence indicates that heme- and flavin-binding domains correspond to similar domains of microsomal cytochrome b_5 and NADH-cytochrome b_5 reductase, respectively. The gene coding the chimeric protein in pCW vector is under bacterial *Taq* promotor.

Expression of chimeric protein cytochrome b_5–NADH-cytochrome b_5 reductase. Overnight culture of recombinant *E. coli* DH5α was diluted in ratio 1 : 500 with TBS-medium (12 g yeast extract, 6 g tryptone, 1 g peptone, 2 ml glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4, and 1 mM thiamine) containing 100 μg/μl ampicillin and incubated under shaking at 37°C till the absorbance at 600 nm (A_{600}) reached 0.3-0.4. Then 5-aminolevulinic acid was added to incubation mixture to final concentration 0.5 mM and incubation was continued at 37°C till absorbance at 600 nm (A_{600}) reached 0.6-

0.8. Expression of the fusion protein cytochrome b_5–NADH-cytochrome b_5 reductase was induced by adding IPTG to final concentration 1 mM, and the culture was carefully incubated at 20°C. After 45 min of incubation, 5-aminolevulinic acid was added to the mixture to final concentration 1.5 mM, and the mixture was incubated for 40-48 h at 20°C.

The expression level of the chimeric protein was approximately 3500 nmol/liter of culture.

Isolation and purification of chimeric protein cytochrome b_5–NADH-cytochrome b_5 reductase from *E. coli* cells. The recombinant cells were collected at 3000 rpm during 10 min. The pellet was suspended at 4°C in 25 mM Tris-HCl buffer (pH 8.0) containing 10% glycerol and 0.5 mM PMSF (buffer A). The cells were broken by ultrasonic treatment using a UZDN-2T (22 kHz, 40% power) during 6-8 cycles for 30 sec each. Disrupted cells were centrifuged at 5000 rpm during 10 min to remove unbroken cells and then at 22,000 rpm during 40 min to collect membrane fraction containing fusion protein cytochrome b_5–NADH-cytochrome b_5 reductase. Membrane fraction was suspended in 25 mM Tris-HCl buffer (pH 8.0). Non-ionic detergent Triton X-100 at final concentration 1% (w/v) was used to solubilize the membranes. Solubilization was carried out under constant mixing during 2.5 h at 4°C. Then solubilized membranes were centrifuged at 22,000 rpm during 40 min.

Further purification was performed using ion-exchange chromatography. Solubilized protein was applied to a DEAE-agarose column preliminarily equilibrated with buffer A containing 0.2% Triton X-100. The column was washed with equilibrating buffer containing 0.1 M NaCl and 0.2% sodium cholate. The fusion protein cytochrome b_5–NADH-cytochrome b_5 reductase was eluted with buffer A containing 0.3 M NaC1 and 0.2% sodium cholate. In the second stage of purification, we used affinity chromatography. Blue-Sepharose 4B that effectively binds NADH-dependent enzymes was used as the affinity matrix. The fractions containing the fusion protein were applied onto a column with Blue-Sepharose 4B preliminarily equilibrated with 25 mM Tris-HCl buffer (pH 8.0) containing 10% glycerol and 0.2% sodium cholate (buffer B). The column was washed with equilibrating buffer (25 mM Tris-HCl) containing 1 M NaCl and 0.2% sodium cholate. The fusion protein was eluted with buffer B containing 10 mM NAD^+, 1 M NaCl, and 0.3% sodium cholate**.**

The concentration of the fusion protein was determined from an absolute absorption spectrum based on the content of cytochrome b_5 using molar extinction coefficient ε_{413} = 124.1 $mM^{-1}\cdot cm^{-1}$ (sum of molar extinction coefficients for cytochrome b_5 (117 $mM^{-1}\cdot cm^{-1}$) and FAD (7.1 $mM^{-1}\cdot cm^{-1}$ at 413 nm)) or from difference spectrum between reduced and oxidized forms of cytochrome b_5 using molar extinction coefficient $\varepsilon_{424\text{-}409}$ = 185 $mM^{-1}\cdot cm^{-1}$.

Determination of enzymatic activity of NADH-cytochrome b_5 reductase in the fusion protein with respect to ferricyanide reduction. To register the reaction kinetics of potassium ferricyanide reduction by the chimeric protein, we used reaction mixture containing 50 mM Tris-HCl (pH 7.4, 20°C), 100 mM NaCl, 30 mM $K_3Fe(CN)_6$, and 10 nM cytochrome b_5–NADH-cytochrome b_5 reductase.

The reaction mixture (total volume 1.4 ml) was placed into a spectrophotometric cell and 50 mM NADH (final concentration 500 μM) was added. After mixing, the change in absorbance at 420 nm ($\varepsilon_{ox} - \varepsilon_{red}$ = 1020 $M^{-1}\cdot cm^{-1}$) was registered. The concomitant reduction of cytochrome b_5 under these experimental conditions changes optical density at this wavelength by less than 0.001 and was not taken into account.

Kinetic measurements. To reduce cytochrome b_5 by NADH-cytochrome b_5 reductase in the chimeric protein, the necessary amount of cytochrome b_5–NADH-cytochrome b_5 reductase was placed in 50 mM Tris-HCl buffer, pH 7.4, the mixture was incubated during 15 min at the desired temperature, and then 10-fold excess of freshly prepared 10 mM NADH in water was added. The changes in absorbance at 424 nm ($\varepsilon_{red} - \varepsilon_{ox}$ = 123,000 $M^{-1}\cdot cm^{-1}$) were monitored. The kinetic curve was constructed on the basis of disappearance of the oxidized form of cytochrome b_5 in the course of the reaction. The concentration of oxidized form of cytochrome b_5 before addition of NADH was accepted as the initial concentration. The concentration of chimeric protein in the sample was determined from the absorbance in the Soret region at 413 nm using molar extinction coefficient for the fusion protein, 124.1 $mM^{-1}\cdot cm^{-1}$.

Determination of pseudofirst order rate constants. The rate constant for cytochrome b_5 reduction by NADH-cytochrome b_5 reductase in the chimeric protein was determined by nonlinear approximation of the kinetic curve for consumption of oxidized cytochrome b_5 during reaction using the equation

$$C_{ox}(t) = C_{ox_0} e^{-k_{obs}t}$$

using the Origin Pro 7.5 program, where $C_{ox}(t)$ is concentration of oxidized cytochrome b_5 at time t (M), C_{ox_0} is concentration of oxidized cytochrome b_5 at the initial time, k_{obs} is the pseudofirst order rate reaction constant (sec^{-1}), and t is time (sec).

Determination of second order rate constants. The rate constant of the reaction of cytochrome b_5 reduction by NADH-cytochrome b_5 reductase in the chimeric protein was determined from the pseudofirst order rate constant using the equation

$$k_2 = k_{obs}/c_{red},$$

where k_2 is the second order rate constant, k_{obs} is the pseudofirst order rate constant for cytochrome b_5, and c_{red} is

concentration of the chimeric protein in the reaction mixture.

Determination of activation energy. To determine the activation energy for the reaction, we first determined second order rate constants at different temperatures (in the range 293-308 K with interval 5 K). To exclude the effect of pH changes at different temperatures, buffer solutions were prepared at the corresponding temperatures. Then the dependence curve $\ln(k_2)$ versus $1/T$ was constructed, where T is thermodynamic temperature. The data were further approximated by the linear function

$$\ln k_2 = A - B\frac{1}{T},$$

and using the least squares method coefficients A and B were determined, where $A = \ln(f)$, $B = E_a/R$, f is the pre-exponential factor in the Arrhenius equation, E_a is activation energy of reaction, R is the gas constant. Activation energy was determined according to equation $E_a = BR$.

Determination of reorganization energy for electron transfer processes. Reorganization energy was calculated according to the Marcus equation:

$$E_a = \frac{(\Delta G + E_p)^2}{4E_p},$$

where E_a is activation energy (J/mol), ΔG is free energy of electron transfer from donor to acceptor (J/mol), and E_p is reorganization energy (J/mol).

Free energy of the electron transfer from donor to acceptor was determined by assuming that the process is reversible, this allowing us to ignore the entropy component in the Gibbs–Helmholtz equation and to equate the free energy to the thermal effect of reaction and obtain equation:

$$\Delta G = -(\varphi_{cytb_5} - \varphi_{cytb_5red})F,$$

where φ_{cytb_5} is electrode oxidation–reduction potential of cytochrome b_5 (0 mV) [29], φ_{cytb_5red} is electrode oxidation–reduction potential of NADH-cytochrome b_5 reductase (–268 mV) [30], and F is the Faraday constant.

The medium reorganization energy for the electron transfer reaction was calculated by solving the quadratic equation, obtained using experimentally determined ΔG and E_a.

Modeling of tertiary structure of chimeric protein cytochrome b_5–NADH-cytochrome b_5 reductase. To construct the model of tertiary structure of the chimeric protein, we used the program for comparative modeling Modeller 8v2 [31], which use the method of "satisfaction of spatial restraints". As a template for construction of the model, we used coordinates for the hydrophilic domain (31 amino acids removed from the N-terminal sequence) of rat liver NADH-cytochrome b_5 reductase (PDB code 1I7P) and hydrophilic domain (94 N-terminal amino acids) of rat liver microsomal cytochrome b_5 (PDB code 1aw3). To check the created model we used the Procheck and Prosa programs. Since the heme and FAD, being the components of template structures, and as expected, domains of the chimeric protein formed by these cofactors, have structures similar to those of individual proteins, we used the inbuilt program function (conformation of heme and FAD was assumed to be rigid) to insert these ligands.

Determination of steady-state fluorescence anisotropy for free FAD and FAD in the chimeric protein. The steady-state fluorescence anisotropy for free cofactor and cofactor bound with the chimeric protein was calculated from fluorescence spectra (λ_{ex} = 450 nm, λ_{em} = 510-580 nm), obtained under four different positions of excitation and emission polarizer using following equation:

$$A = \frac{I_{\parallel} - kI_{\perp}}{I_{\parallel} + 2kI_{\perp}},$$

where A is steady-state anisotropy, $I_{\parallel}$ is intensity of parallel polarized component of fluorescence under vertical polarization of the excitation light, $I_{\perp}$ is intensity of perpendicularly polarized component of fluorescence under vertical polarization of the excitation light, and k is the gauge factor. All measurements were done at 20°C.

The rotational correlation time for the fluorophore was calculated using the Perrin equation [32, 33]:

$$A^{-1} = A_0^{-1}\left(1 + \frac{t_F}{t_c}\right),$$

where A is steady-state anisotropy, A_0 is fundamental steady-state anisotropy, t_F is fluorescence lifetime, and t_c is the rotational correlation time.

The fundamental steady-state anisotropy of FAD was determined by measuring steady-state anisotropy of the FAD solution (5 mM) of known viscosity (glycerol solution used to calibrate viscosimeters) and extrapolating the Perrin plot to zero T/η ratio.

RESULTS AND DISCUSSION

Figure 2 shows the absorption spectra of highly purified recombinant fusion protein consisting of the hydrophilic fragment of cytochrome b_5 and the truncated form of rat liver NADH-cytochrome b_5 reductase (23 amino acid residues deleted from the N-terminal hydrophobic membrane-binding sequence of the flavoprotein). The chimeric protein contains in a single polypeptide chain cytochrome b_5 (94 amino acids) and the 23 amino acid N-terminal truncated NADH-

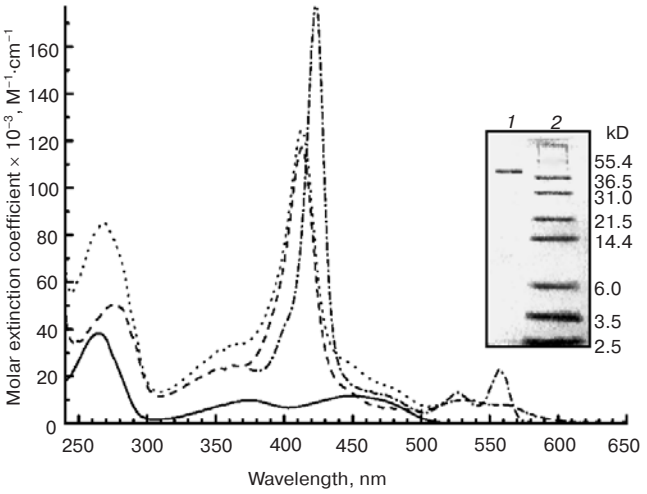

Fig. 2. Absolute absorption spectrum of FAD (solid line), cytochrome b_5 oxidized form (dashed line), and highly purified fusion protein cytochrome b_5–NADH-cytochrome b_5 reductase (oxidized and reduced forms) (dotted and dash-dotted lines, respectively). Inset: SDS-PAGE in 13% gel: *1*) highly purified fusion protein cytochrome b_5–NADH-cytochrome b_5 reductase; *2*) standard proteins Mark12™.

cytochrome b_5 reductase (277 amino acids), which are linked via the spacer group Ser-Thr-, which is also necessary to introduce the *Sal* I restriction site (377 amino acids). High purification degree of the fusion protein and correspondence according to molecular weight to that expected for the chimeric protein (42.8 kD) are confirmed by results of SDS-PAGE (Fig. 2, inset).

The absolute absorption spectrum of the fusion protein cytochrome b_5–NADH-cytochrome b_5 reductase is very similar to the absorption spectrum of the oxidized form of cytochrome b_5 and has maximum of absorbance in the Soret region at 413 nm and two less evident peaks in the long-wavelength region of the spectrum. The presence of the flavoprotein component is confirmed by the broad shoulder in the region of 450 nm, which is characteristic for absorbance of the flavin cofactor of NADH-cytochrome b_5 reductase. Reduction of cytochrome b_5 with sodium dithionite results in dramatic change of the absorption spectrum (Fig. 2). The absorption maximum in the Soret region is greatly increased and shifts to 424 nm, the shoulder at 450 nm disappears, and in the long-wavelength region two peaks at 527 and 557 nm appear, which is characteristic for cytochrome b_5.

NADH-cytochrome b_5 reductase in the fusion protein is able to reduce ferricyanide-anion with second order rate constant about $4 \cdot 10^5$ $M^{-1} \cdot sec^{-1}$, which corresponds to activity of 8.5 nmol of substrate/nmol enzyme per second.

A characteristic feature of the fusion protein is its ability in the presence of exogenous electron donor, NADH, to reduce cytochrome b_5, the second component of the fusion protein, which is indicated by change of the absorption spectrum of the protein. Cytochrome b_5 not in the chimeric protein is not able to be reduced by NADH [1]. Not having experimental data relative to mutual localization of protein globules in the chimeric protein and correspondingly with respect to localization of the cofactor, it is possible to propose two hypotheses on possible pathways of electron transfer from NADH-cytochrome b_5 reductase to cytochrome b_5: (i) intermolecular electron transfer between cytochrome b_5 of one molecule of fusion protein and NADH-cytochrome b_5 reductase of another; (ii) intramolecular electron transfer within single chimeric protein molecules.

The second hypothesis presumes special orientation of the globules of the interacting proteins resulting in close location of the active sites. The distance between donor and acceptor necessary for electron transfer is usually accepted to be 5-20 Å. It is reasonable to suggest that in the second case "intramolecular" electron transfer is a monomolecular reaction controlled only by kinetic mechanism. Such reaction should fit the equation kinetics of a first order reaction relative to concentration of the chimeric protein.

The first hypothesis presumes bimolecular reaction of second order, being a reaction of the first order with respect to cytochrome b_5 concentration and first order reaction with respect to NADH-cytochrome b_5 reduc-

Table 1. Dependence of reaction half-life on initial protein concentration (data calculated from kinetic curves)

Concentration of oxidized cytochrome b_5 in the chimeric protein (c_0), nM	Half-life of oxidized cytochrome b_5 in the chimeric protein ($t_{1/2}$), sec	Pseudofirst order reaction rate constant (reduction of cytochrome b_5 by reductase) calculated from reaction half-life, $\ln(2)/t_{1/2}$, sec^{-1}
146	93 ± 1	0.0074 ± 0.00008
130	95 ± 1	0.0072 ± 0.00007
122	91 ± 2	0.0076 ± 0.00016
81	97 ± 2	0.0103 ± 0.00015
65	96 ± 3	0.0072 ± 0.00023

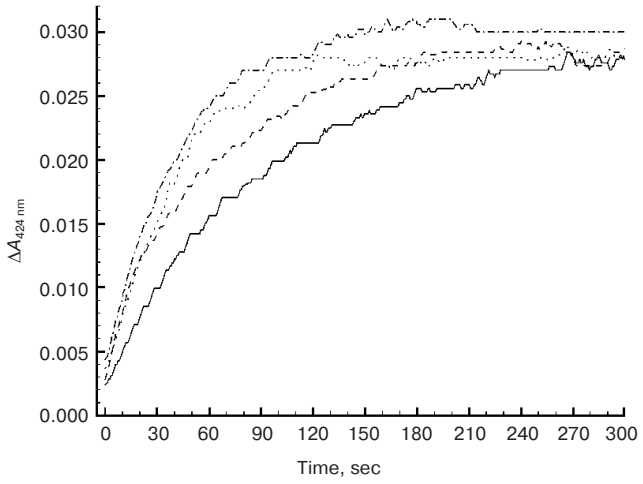

Fig. 3. Kinetic curves of reduction of cytochrome b_5 in the chimeric protein by NADH-cytochrome b_5 reductase, obtained by monitoring changes in optical density at 424 nm. On the Y-axis the change of optical density at 424 nm, which corresponds to cytochrome b_5 reduction. The kinetic curve was approximated by function $f(x) = C_0 (1 - e^{-kx})$, where C_0 and k were obtained by the least squares method. The initial concentration of the fusion protein was 0.564 nM. Only the registered part of the kinetic curve is shown in the figure; the initial part was lost during mixing of the components. The reaction was conducted in 50 mM Tris-HCl buffer, pH 7.4, at 293 K with various concentrations of sodium chloride: 0 mM (solid line); 50 mM (dashed line); 100 mM (dotted line); 150 mM (dash-dotted line). The reaction was started by addition of 10 mM NADH solution to final concentration 100 μM.

tase. However, since the reaction mixture contains a large excess of NADH, the concentration of NADH-cytochrome b_5 reductase remains practically constant due high rate of reduction, and thus reaction becomes indeed a first order reaction with respect to cytochrome b_5. Using the fractional time method, it was shown that the half-life of oxidized cytochrome b_5 does not depend on its initial concentration (Table 1). Such dependence is characteristic for reactions having first order with respect to a chemical whose change of concentration is illustrated on the kinetic curve (Fig. 3).

Since we were mainly interested in the reaction of electron transfer from FAD of NADH-cytochrome b_5 reductase to heme of cytochrome b_5, we excluded from the reaction the electron transfer from NADH to FAD by increasing the concentration of NADH in reaction mixture to 200-fold excess of NADH relative to protein. At 100 μM NADH, the electron transfer from FAD to heme becomes the rate-limiting step of the overall reaction (Fig. 4).

The studies of the dependence of the first order rate constant with respect to cytochrome b_5 concentration on the initial concentration of chimeric protein (which is numerically equivalent to the concentration of the reduced NADH-cytochrome b_5 reductase) have shown that the reaction is a bimolecular reaction of pseudofirst order, the rate constant of which is equal to the product of second order rate constant and the chimeric protein concentration. For calculation of the second order rate constant, it is necessary to know the pseudofirst order rate constant with respect to cytochrome b_5 and concentration of chimeric protein in solution. Analysis of our kinetic curves indicates that cytochrome b_5 reduction by NADH-cytochrome b_5 reductase in the fusion protein is a bimolecular reaction, and in the elementary act of electron transfer two molecules of the chimeric protein participate (Table 2). This assumption means that the chimeric protein should reduce also exogenous cytochrome b_5, which was proved experimentally. Addition of exogenous cytochrome b_5 to reaction mixture containing completely reduced cytochrome b_5 in the fusion protein results in reduction of exogenous cytochrome b_5, which is easily observed by changes of absorbance at 424 nm. The rate constant of the bimolecular reaction of exogenous cytochrome b_5 reduction is close to the corresponding rate constant for reduction of cytochrome b_5 in the chimeric protein.

To investigate the relative position of the heme of cytochrome b_5 and FAD of NADH-cytochrome b_5 reductase in the fusion protein, we built a model of the tertiary structure of the chimeric protein using the Modeller 8v2 program (Fig. 5, see color insert). As follows from the tertiary structure model, the distance between the isoalloxazine ring of FAD (electron donor) and heme iron (electron acceptor) is 50 Å. It is known that overlapping of molecular orbitals is necessary for electron transfer, and the distance from donor to acceptor of electrons must be 5-20 Å [23]. Thus, according to the model of the tertiary structure for the chimeric protein, intramolecular electron transfer in the fusion protein is not possible.

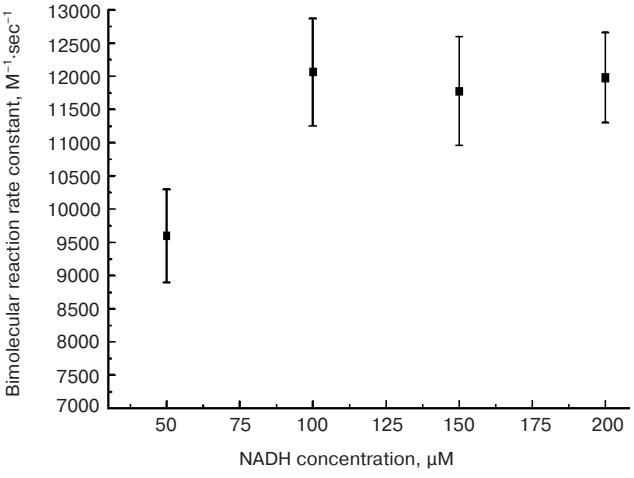

Fig. 4. Dependence of bimolecular rate constant on NADH concentration. The reaction was conducted in 50 mM Tris-HCl buffer, pH 7.4, at 293 K.

Table 2. Determination of reaction order for cytochrome b_5 reduction by NADH-cytochrome b_5 reductase in the chimeric protein from the pseudofirst order rate constants

Initial concentration of chimeric protein, nM	Pseudofirst order rate constant on cytochrome b_5, sec^{-1}	Second order rate constant (k_2), $M^{-1}\cdot sec^{-1}$
267	0.0033 ± 0.00004	12 360 ± 150
564	0.0068 ± 0.00007	12 060 ± 109
1132	0.0146 ± 0.00012	12 897 ± 106

Table 3. Dependence of rate constant, pre-exponential factor in the Arrhenius equation, activation energy, and reorganization energy on sodium chloride concentration in the reaction mixture

Sodium chloride concentration, mM	Second order rate constant (293 K), $M^{-1}\cdot sec^{-1}$	ln$A$, $M^{-1}\cdot sec^{-1}$	Activation energy, kJ/mol	Reorganization energy, kJ/mol
0	13490 ± 117	34 ± 4	60 ± 6	277 ± 19
50	13851 ± 145	32.05 ± 0.08	54.8 ± 0.2	256 ± 19
100	19189 ± 178	21.6 ± 2.4	28 ± 6	150 ± 20
200	25245 ± 269	21.5 ± 2.7	27 ± 7	147 ± 21
300	28014 ± 322	21.5 ± 3	27 ± 0.2	144 ± 15

Comparison of the rate constant for reduction of cytochrome b_5 in the chimeric protein at different concentrations of sodium chloride in the reaction mixture shows increase of reaction rate constant with increasing salt concentration and ionic strength (Table 3). A similar effect was found earlier for some other reactions of electron transfer, for example, NADPH-cytochrome P450 reductase and cytochrome b_5, NADPH-cytochrome P450 reductase and cytochrome c, as well as for some other redox proteins, and this was explained mainly based on the concept of the role of diffusion in electron transfer. To explain this effect, several hypothesis have been suggested [27, 34-36]: (i) the ions mask similar charges on the interacting surfaces of the protein globules (theory of repulsions); (ii) the ions facilitate dissociation of the electron donor (NADH in the case of NADH-cytochrome b_5 reductase or NADPH in the case of NADPH-cytochrome P450 reductase); (iii) the increasing of solution ionic strength facilitates close contact between flavin and heme due to increase of hydrophobic interactions; (iv) the increasing of solution ionic strength facilitates dissociation of protein–protein complex after reaction; (v) the increasing of solution ionic strength facilitates dissociation of protein aggregates.

If one of these hypotheses is valid and the reaction rate is indeed determined either by diffusion or interaction between molecules, i.e. is under diffusion control, this means that addition of salt ions to the reaction mixture should not affect the activation energy of the oxidation–reduction reaction. In this case, for two particular conditions the Arrhenius plots should in the ideal case be parallel lines.

In the case when the reaction is kinetically controlled and changes in rate constants are determined by different activation barriers, curves of Arrhenius plots should intersect in a single point located on the coordinate axis.

Thus, to understand the real mechanism of the effect of ions on the bimolecular rate constant of electron transfer, we determined the values of the activation barriers. As shown, the increase of ion concentration in solution dramatically decreases activation energy of reaction. This means that first of all it is necessary to understand the reason why addition of ions decreases activation barrier.

The nature of the activation barrier in electron transfer reactions is explained by Marcus theory for electron transfer in polar media [23]. According to this theory, the main reason for appearance of energy activation in solution is orientational polarization of the medium, or anisotropy in position of the solvent molecules in the vicinity of the forming pair donor–acceptor components. Initial reagents and reaction products have different equilibrium medium polarization, or unequal distribution of solvent molecules. For electron transfer it is necessary that the medium is correlated with reaction products. By the way, for electron transfer to occur a necessary condition is reorganization and reconstruction of the medium.

The energy necessary for that process is called the energy of medium reorganization and its physical sense represents the energy needed to fit medium to reaction products, but the electron should stay in the initial position. It is evident that the main reason for the appearance of activation energy in the reaction of electron transfer between cytochrome b_5 and NADH-cytochrome b_5 reductase is the presence of water molecules in the region of sites of protein interaction. Increasing salt concentration in the reaction mixture results in replacement of water molecules by ions, which results in decrease of the energy of medium reorganization and, correspondingly, to decrease of activation energy.

Experimental results confirmed this assumption completely. The Arrhenius plots for different sodium chloride concentrations are shown in Fig. 6. Experimentally determined values of bimolecular reaction rate constants, logarithm of pre-exponential factor in the Arrhenius equation, reaction activation energy, as well as reorganization energies calculated on the assumption that the process is reversible are presented in Table 3. It is necessary to consider the anomalously high values of the pre-exponential factor for rate constants at low ionic strength of solution and their sharp decrease with increasing anion concentration in the reaction mixture.

Assuming that every molecular collision results in interaction and using the transformed for two similar molecules equation for the rate constants of the second order reaction controlled by diffusion, it is possible to estimate the approximate value of the maximal significance of the pre-exponential factor:

$$k = 8000\ \pi r_0 D N_a,$$

where k is the rate constant of the bimolecular reaction controlled by diffusion ($M^{-1}\cdot sec^{-1}$), r_0 is minimal distance between centers of the molecules reached under closing, D is the translational diffusion factor ($m^2\cdot sec^{-1}$), and N_a is Avogadro's number.

It is possible to calculate the diffusion coefficient for the chimeric protein by using the Stokes—Einstein equation:

$$D = \frac{kT}{6\pi\eta r},$$

where D is translational diffusion factor ($m^2\cdot sec^{-1}$), k is the Boltzmann constant ($J\cdot K^{-1}$), T is the thermodynamic temperature (K), η is the dynamic viscosity of the medium (Pa·sec), and r is average hydrodynamic radius of the molecule (m).

The pre-exponential factor, under conditions when every collision results in formation of protein—protein complex ready to perform electron transfer, was calculated using the Smoluchowski equation [37] for diffusion controlled reactions and has the value of about 10^{10}.

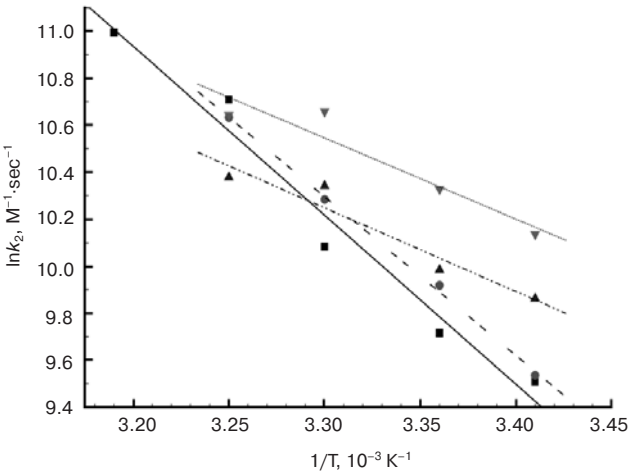

Fig. 6. Arrhenius plots of cytochrome b_5 reduction reaction in the chimeric protein with different sodium chloride concentrations in the reaction mixture: 0 mM (solid line, squares); 50 mM (dashed line, circles); 100 mM (dash-dotted line, triangles); 200 mM (thin line, inverted triangles).

Experimentally determined pre-exponential factor has the order value about 10^{15}. It is evident that one of the reasons for such anomalously high value of pre-exponential factor might be the fact that after addition of NADH to the reaction mixture, equilibrium in the system is setup and cytochrome b_5 forms complex with NADH-cytochrome b_5 reductase in accordance with the value of dissociation constant characteristic for that pair of redox proteins. Also, electrostatic interactions between molecules might be the reason for elevated values of bimolecular reaction rate constants controlled by diffusion by several orders of magnitude. It is necessary to stress that the character of intermolecular interactions is mainly related to types monopole—dipole and dipole—dipole, since the interaction involves similar molecules. For the constructed model of the tertiary structure of the chimeric protein as well as for the structures of hydrophilic domains of cytochrome b_5 (1AW3) and NADH-cytochrome b_5 reductase (1I7P) using the WEB-server Protein Dipole Moment Server (PDMS, http://bip.weizmann.ac.il/dipol) [38], we calculated the dipole moments. The value for the model proved to be significant and comprised 1075 D. For comparison, the dipole moment for the hydrophilic domain of cytochrome b_5 is 514 D, while for the hydrophilic domain of NADH-cytochrome b_5 reductase is 861 D.

The dependence of the pre-exponential factor on the ionic strength of the solution can be described by the Debye—Huckel theory of ionic solutions. Quantitative prediction of the value of activation barrier is a very complicated task that requires significant calculations and additional experimental results. However, it is possible to make a qualitative estimation.

As follows from the Arrhenius plots presented in Fig. 6, the rate constant is determined at a particular temperature both by the pre-exponential factor and the activation energy. At temperatures higher than the temperature corresponding to the intersection of the Arrhenius plots for different concentration of ions in solution, the effect of decrease of the activation energy on the decrease of medium reorganization energy will be negligible, while the main role will be played by pre-exponential factor; therefore, the rate constant for such reaction will be higher at the same temperature in the system with low salt concentration.

Vice versa, at the lower temperature the effect of activation energy on value of the rate constant will prevail as compared to the effect of the pre-exponential factor. Consequently, the effect of ions present in the media in which electron transfer takes place with participation of protein globules depends on temperature, quantity of water at the region of the binding site of electron donor and acceptor, and difference in oxidation–reduction potentials of donor and acceptor. The point of intersection of the Arrhenius plots for two different conditions can be called the point of equivalence, since at that point the rate constants are the same. By using this definition, the abovementioned may be formulated in the following way: (i) at temperatures higher than the temperature corresponding to the point of equivalence, the difference in rate constants is connected with the fact that the reaction is controlled by diffusion; (ii) at temperatures lower than the temperature corresponding to the point of equivalence, the difference in rate constants is connected with kinetic control of the reaction rate.

Thus, based on our data it is possible to predict the behavior of the rate constant in dependence on the presence of anions or other molecules in the reaction medium. For simple systems, for which electron transfer is not followed by changes of internal coordination sphere (anions, simple molecules), the energy of medium reorganization can be calculated using the equation [26]:

$$E_s = (\frac{1}{\varepsilon_\infty} - \frac{1}{\varepsilon_0})e^2(\frac{1}{2r_a} + \frac{1}{2r_d} - \frac{1}{R}),$$

where ε_∞ is the optical dielectric constant of the solvent, ε_0 is the static dielectric constant of the solvent, e is the electron charge, r_a is the radius of the electron acceptor, r_d is the radius of electron donor, and R is the distance between centers of the electron donor and acceptor.

This equation, however, is not applicable for electron transfer reaction from FAD to Fe^{3+} of heme since the molecules are complex and electron transfer reaction is usually followed by reorganization not only outside the sphere (solvent molecules), but also in internal atomic coordinates. However, despite that it is possible to use this simplified model for qualitative prediction of the behavior of the system in different media.

Table 4. Comparison of dielectric constants for water and glycerol

Solvent	ε_∞	ε_0	$\frac{1}{\varepsilon_\infty} - \frac{1}{\varepsilon_0}$
Water	1.77	80	0.553
Glycerol	2.161	30	0.428

It is evident that effect of the medium in which the reaction takes place is determined by factor

$$(\frac{1}{\varepsilon_\infty} - \frac{1}{\varepsilon_0}).$$

The data in the table of permittivity for water and glycerol as well as the value of the indicated factor are presented in Table 4. It is reasonable to expect that inclusion of glycerol in the reaction mixture should decrease activation energy of the reaction of electron transfer. However, decrease of energy activation for glycerol should be much less significant since the glycerol molecule is much bigger than ions and does not carry charge. To check this assumption, we did corresponding experiments. Arrhenius plots for reaction of electron transfer are shown in Fig. 7, while the data of measurements and corresponding calculations are indicated in Table 5. In accordance with this assumption, activation energy is decreased. Simultaneously decreased of the value of the pre-exponential factor appears to be caused by the fact that glycerol facilitates dissociation of the complex of

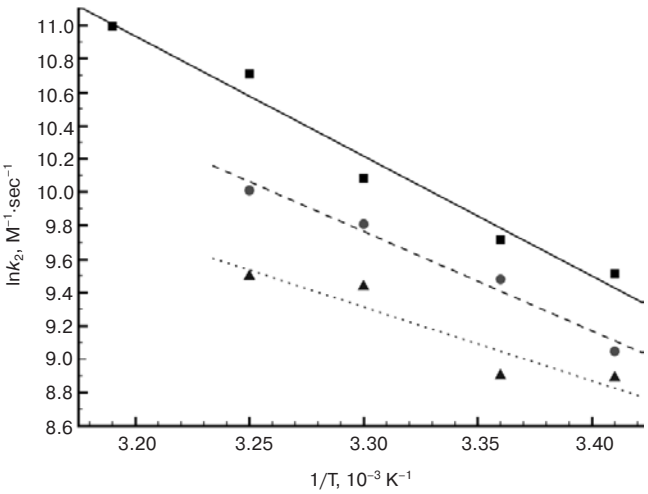

Fig. 7. Arrhenius plots of cytochrome b_5 reduction reaction in the chimeric protein with different glycerol concentrations in the reaction mixture: 0% (solid line); 5% (dashed line); 15% (dotted line).

Table 5. Dependence of rate constant, pre-exponential factor A in the Arrhenius equation, activation energy, and reorganization energy on glycerol concentration in the reaction mixture

Glycerol concentration, %	Second order rate constant (293 K), $M^{-1}\cdot sec^{-1}$	ln$A$, $M^{-1}\cdot sec^{-1}$	Activation energy, kJ/mol	Reorganization energy, kJ/mol
0	13490 ± 117	34 ± 4	60 ± 6	277 ± 19
5	8488 ± 85	28 ± 2	48 ± 5	230 ± 16
15	7245 ± 198	23 ± 4	35 ± 10	177 ± 23

redox partners or prevents its formation. The decrease of the value of pre-exponential factor in this case can be explained by increase of medium viscosity after increasing glycerol concentration. It is found that glycerol at relatively high concentrations (10% w/w) facilitates dissociation of protein complexes in the chimeric protein. Thus, relatively small decrease of energy of activation of the reaction together with decrease of the value of pre-exponential factor in the Arrhenius equation is the real reason for the experimentally observed decrease of reaction rate constant. It is necessary to stress that the effect of ionic strength on the reaction rate constants with participation of heme proteins such as cytochrome *c* and the set of transition metal compounds was studied and interpreted in the frame of the Debye–Huckel theory [39, 40]. The studies also show the effect of dipole moment of protein molecules on intermolecular interactions. However, the results of kinetic studies of cytochrome P450-dependent systems were interpreted in a different way, although they appear to be explained from the same positions.

The state in which the chimeric protein exists in solution is of special interest. Since the binding centers of the proteins are not sterically hidden, it is possible to presume the formation in solution of oligomeric complexes of "head-to-tail" type. To determine the state in which the chimeric protein exists in solution, we used fluorescence of cofactor FAD of NADH-cytochrome b_5 reductase. Fluorescence spectra of FAD bound with the chimeric protein are shown in Fig. 8. The fundamental steady-state FAD anisotropy determined from Perrin plots is 0.4. These results of measurement of steady-state anisotropy are presented in Fig. 8 (inset). The steady-state anisotropy of FAD in 50 mM Tris-HCl buffer, pH 7.4, is 0.027, while that for FAD in the chimeric protein is 0.082. It is known that the FAD fluorescence lifetime is 2.3 nsec [41]. The rotational correlation time for free FAD calculated from the Perrin equation is 0.166 nsec. For FAD bound with a protein molecule the fluorescence lifetime usually varies and is in the interval from 0.1 to 5 nsec [42]. This allows determination of the interval for the rotational correlation time for FAD bound with protein. We found that this interval is in the range 0.025-1.289 nsec. Since FAD bound with protein cannot be more flexible than free flavin in solution, it is reasonable to think that rotational correlation time for FAD bound with chimeric protein should be in the interval 0.166-1.289 nsec, while the time interval is 0.6-5 nsec. By using the equation for rotational diffusion and taking into account that the form of hydrated molecules is close to the form of a sphere, it is possible calculate the radius of the molecule. For FAD this value is 5.44 Å, while the distance between the farthest atoms of the isoalloxazine ring of FAD is 10.1 Å. In the same way, it is possible to determine the interval in which the radius of hydrated protein globule lies: 5.3-10.8 Å. The mean radius of the NADH-cytochrome b_5 reductase globule is 25 Å, while the rotational correlation time calculated for the indicated conditions is about 13 nsec. Comparison of these values indicates that the FAD cofactor not rigidly bound with the protein molecule and has relatively high mobility. Finally, the experimentally determined time of rotational correlation for FAD is much less compared to theoretically calculated ones.

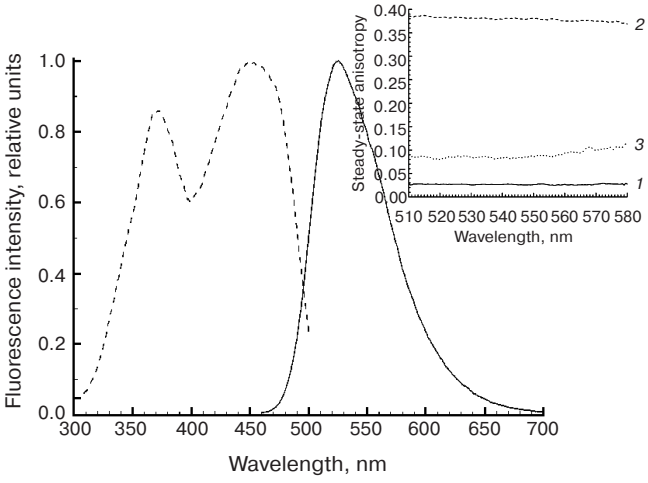

Fig. 8. Fluorescence spectra of chimeric protein cytochrome b_5–NADH-cytochrome b_5 reductase: excitation spectrum (dashed line); emission spectrum (solid line). Inset: steady-state anisotropy of free FAD (3 mM FAD in 50 mM Tris-HCl, pH 7.0 (solid line, *1*), and 3 mM FAD in 99% glycerol (dashed line, *2*)) and FAD bound with the protein (3 mM FAD with 1 mM cytochrome b_5–NADH-cytochrome b_5 reductase in 50 mM Tris-HCl, pH 7.0 (dotted line, *3*)).

We proposed that formation and decay of intermolecular protein complexes of the chimeric protein should result in changes of time of rotational correlation for FAD bound with the protein globule and correspondingly to changes of stationary fluorescence anisotropy for FAD. Since it is known that cytochrome b_5 and NADH-cytochrome b_5 reductase form a complex stabilized by electrostatic interactions, the increase of ionic strength of solution resulting in dissociation of protein complexes should result in decrease of steady-state fluorescence anisotropy. To prove this, we did some experiments. It was found that on increasing sodium chloride concentration in solution from 0 to 350 mM, the fluorescence anisotropy is insignificantly increased from 0.082 to 0.095, and the reason for this appears to be the increase of solution viscosity. Inclusion into solution of detergent (sodium cholate) does not change the steady-state anisotropy of FAD. Consequently, we failed to prove the presence of protein complexes of chimeric protein using steady-state fluorescence anisotropy of FAD.

Thus, in the present work, based on kinetic analysis of cytochrome b_5 reduction in the fusion protein cytochrome b_5–NADH-cytochrome b_5 reductase, we have shown that in the elementary process of electron transfer at least two molecules of redox proteins belonging to different chimera molecules are involved. As follows from the tertiary structure of the chimeric protein (Fig. 5), the distance between electron donor (FAD) and electron acceptor (heme) is more than 50 Å, indicating inability of electron transfer within a single chimeric molecule.

The comparison of experimentally determined values of steady-state fluorescence anisotropy and values from corresponding theoretical calculations have shown that FAD, being tightly bound with protein globule, becomes more flexible relative to the polypeptide chain of NADH-cytochrome b_5 reductase, which may be a result of intramolecular protein dynamics. The finding of stable dimer complexes of the chimeric protein using fluorescence polarization of the fluorescent marker FAD proved to be complicated due to significant mobility of the cofactor with respect to the protein globule.

The electron transfer reaction of chimeric protein molecules may be controlled both by diffusion and kinetically depending on the character of the medium where it occurs. Increasing ionic strength of solution results in decrease of the efficiency of protein–protein interactions and simultaneously to decrease of activation energy.

Thus, we for the first time carefully studied and explained the effect of ions and solved polar compounds present in the reaction mixture on the value of rate constant of electron transfer reaction with participation of cytochrome b_5 and NADH-cytochrome b_5 reductase and have shown that increase of reaction rate constant for electron transfer between NADH-cytochrome b_5 reductase and cytochrome b_5 in the fusion protein is the consequence of the decrease of medium energy reorganization resulting in decrease of energy of the oxidation–reduction reaction.

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