Biochemistry (Moscow), Vol. 68, No. 9, 2003, pp. 994-1001. Translated from Biokhimiya, Vol. 68, No. 9, 2003, pp. 1216-1225.

Chemical Modification of Catalytically Essential Functional Groups of NAD-Dependent Hydrogenase from *Ralstonia eutropha* H16

T. V. Tikhonova[1]*, N. D. Savel'eva[2], and V. O. Popov[1]

[1]Bach Institute of Biochemistry, Russian Academy of Sciences, Leniniskii pr. 33, Moscow 119071, Russia; fax: (7-095) 952-0801; E-mail: ttikhonova@inbi.ras.ru
[2]Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7/2, Moscow 117811, Russia; fax: (7-095) 135-6595; E-mail: ivanov@inmi.host.ru

Received September 17, 2002
Revision received October 25, 2002

Abstract—Amino acid residues His and Cys of the NAD-dependent hydrogenase from the hydrogen-oxidizing bacterium *Ralstonia eutropha* H16 were chemically modified with specific reagents. The modification of His residues of the nonactivated hydrogenase resulted in decrease in both hydrogenase and diaphorase activities of the enzyme. Activation of NADH hydrogenase under anaerobic conditions additionally modified a His residue (or residues) significant only for the hydrogenase activity. The rate of decrease in the diaphorase activity was unchanged. The modification of thiol groups of the nonactivated enzyme did not affect the hydrogenase activity. The effect of thiol-modifying agents on the activated hydrogenase was accompanied by inactivation of both diaphorase and hydrogenase activities. The modification degree and changes in the corresponding catalytic activities depended on conditions of the enzyme activation. Data on the modification of cysteine and histidine residues of the hydrogenase suggested that the enzyme activation should be associated with significant conformational changes in the protein globule.

Key words: NAD-dependent hydrogenase from *Ralstonia eutropha* H16, chemical modification, hydrogenase activity, diaphorase activity

The NAD-reducing hydrogenase from the hydrogen-oxidizing bacterium *Ralstonia eutropha* H16 is a heterotetrameric multifunctional enzyme of the Ni-Fe hydrogenase class. The hydrogenase molecule from *R. eutropha* is functionally subdivided into two independent heterodimers, the hydrogenase heterodimer (HoxHY) on which the reaction $H_2 = 2H^+ + 2e^-$ occurs and the diaphorase (HoxFU) heterodimer which displays NADH-dehydrogenase (diaphorase) activity, $NAD^+ + H^+ + 2e^- = NADH$ [1-4].

Similarly to the well-studied enzyme from the sulfate-reducing bacteria *Desulfovibrio gigas*, the hydrogenase dimer (HoxHY) consists of two subunits with molecular weight of 56 kD (HoxH-subunit) and 26 kD (HoxY-subunit). On the large subunit, a hydrogen-activating site is located which contains a binuclear Ni-Fe-complex in which the Fe ion is bound to several diatomic ligands CO and CN^- [4-8]. On the C-terminus of the large subunit a Mg(II) ion seems to be located (as shown for the enzyme from *Desulfovibrio vulgaris* [9]). The role of this ion in the functioning of hydrogenases is unclear. Unlike the hydrogenase from *D. gigas*, which contains three Fe-S-sites, the small HoxY-subunit has a single [4Fe-4S]-cluster that is an acceptor of electrons produced on splitting of the hydrogen molecule [2]. According to a recent hypothesis [10], an additional site for FMN binding may be located on the HoxY-subunit. No other prosthetic groups were found on the hydrogenase dimer.

The diaphorase, or the HoxFU-heterodimer, contains FMN, the binding site for NAD(H), and several iron-sulfur clusters which seem to have the following composition: one [2Fe-2S]-cluster, two or three [4Fe-4S]-clusters, and, possibly, one [3Fe-4S]-cluster [1, 2, 4, 11].

The literature on the structure and action mechanisms of classical dimeric Ni-Fe-hydrogenases indicates several groups of amino acid residues which are signifi-

Abbreviations: DPC) diethylpyrocarbonate; DTNB) 5,5′-dithiobis(2-nitrobenzoic acid), disodium salt; FC) K^+ ferricyanide; FMN) flavin mononucleotide; IAA) iodoacetamide.

* To whom correspondence should be addressed.

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cant for catalysis and maintaining the native structure of enzymes of this class. First, these residues include Cys, His, and also Glu and Asp.

Residues Glu and Asp are thought to play an important role in formation of the channel for proton transport from the hydrogen-activating site of hydrogenases [5, 12, 13] and also in the coordination of Mg(II) [9, 13].

The more important role in the catalytic action of hydrogenases is ascribed to His residues. X-Ray crystallography data show that in hydrogenases from *D. gigas* [5] and *D. vulgaris* [9] histidine plays the key role in a number of processes. One of the His residues of the small subunit (His185 in the enzyme from *D. gigas* [5] and His188 in that from *D. vulgaris* [9]) is supposed to be a terminal element in the chain of electron transfer from the hydrogen-splitting site to the acceptor; three highly conservative histidines of the hydrogen-splitting subunit which corresponds to the HoxH-subunit of the enzyme from *R. eutropha* may be involved in the proton transfer from the active site to the surface of the molecule [5]. One of the His-residues of this subunit (His552 in hydrogenase from *D. vulgaris*) is a potential ligand of Mg(II) [9].

The primary structure of the HoxH-subunit of hydrogenase from *R. eutropha* contains five structural motifs conservative for Ni-Fe-hydrogenases [14]. Four of them include His residues: His16, His69, His111, His396, and His464 [15, 16]. By similarity to classical hydrogenases, three of them (His69, His396, His464) and possibly His16 are supposed to be involved in the proton transport. The function of His111 is suggested to be associated with stabilization of the C-terminal region of the molecule. The residue His464 homologous to His552 of the hydrogenase from *D. vulgaris* can also be involved in coordination of the Mg(II) [15, 16].

There is no definite data on the role of His residues in functions of the diaphorase subunit of hydrogenase from *R. eutropha*. According to data of [11], the binding sites for [2Fe-2S]- and [4Fe-4S]-clusters and also the binding sites for FMN and NAD contain no His residues in the enzyme with a similar structure from *Anabaena variabilis*.

Thiol groups of Cys play a fundamental role in coordination of various prosthetic groups in hydrogenase molecules, including the coordination of the hydrogen-activating Ni-Fe-site [5, 7, 9, 13]. In molecule of the hydrogenase from *R. eutropha* the Ni-Fe-active center is coordinated by four thiol groups: Cys62, Cys65, Cys458, and Cys461 [15]. Similarly to the enzyme from *D. gigas*, one of the Cys-ligands of the Ni ion (Cys458) is supposed to be a primary acceptor of the proton generated during the heterolytic splitting of the H–H-bond [15].

The small HoxY-subunit of the hydrogenase from *R. eutropha* contains nine Cys residues, some of which are located homologically in the conservative motif on the C-terminus of the molecule and can form a cluster to coordinate the single Fe-S-center [2]. The Cys residues on the diaphorase heterodimer can play a similar role of ligands in the coordination of four iron–sulfur clusters. As follows from the literature data, the role of the various amino acid residues in the functioning of the hydrogenase from *R. eutropha* remains unclear.

Site-directed mutagenesis is a direct approach for studies of catalytically significant amino acid residues. In the laboratory of Prof. B. Friedrich, a number of mutants were prepared with substitutions in five conservative motifs of the HoxH-subunit [15, 16]. However, the data obtained have shown that the substitution of presumably catalytically significant groups often results in disorders in the synthesis and assemblage of the tetrameric molecule of the protein, and this makes it difficult to assess functions of the corresponding residue in the end product.

Chemical modification with specific reagents is an approach used for studies on the role of various functional groups in catalysis and structural organization of the enzyme. This approach also allows us to determine the availability of catalytically significant groups in different redox states of the enzyme and thus to assess its conformational mobility.

The purpose of the present work was to study by chemical modification the role of His and Cys residues in the functioning of the NAD-dependent hydrogenase from *R. eutropha* H16.

MATERIALS AND METHODS

Hydrogenase preparations with the specific activity of 20-30 units/mg (30°C) in NAD reduction with hydrogen were used. The enzyme isolation and purification were performed as previously described [17, 18]. Purity of the enzyme preparations was not less than 90% according to SDS-polyacrylamide gel electrophoresis. The hydrogenase concentration was calculated from the A_{280} value, with the molar extinction coefficient ε_{280} = 250,000 $M^{-1}\cdot cm^{-1}$. Thus calculated, the hydrogenase concentration in the reactions of modification was 1-5 μM.

The following reagents were used: NAD (Sigma, USA) with NAD content of 96%; NADH (Reanal, Hungary) with NADH content of 80% and (Boehringer, Germany, grade 1) with the NADH content of 100%; potassium ferricyanide (FC), pure for analysis (Reakhim, Russia); diethylpyrocarbonate (DPC), iodoacetamide (IAA) (Sigma); 5,5′-dithiobis(nitrobenzoate) (DTNB) (Serva, Germany); and gaseous hydrogen with the total content of oxygen and nitrogen no more than 0.01%.

Determination of the hydrogenase activity. The enzyme activity in reduction of NAD and FC with molecular hydrogen was determined spectrophotometrically as described in [18] by monitoring of NADH produced (ε_{340} = 6220 $M^{-1}\cdot cm^{-1}$) or decrease in the concentration of oxidized FC (ε_{420} = 1220 $M^{-1}\cdot cm^{-1}$).

The hydrogenase activity in the diaphorase reduction of FC in the presence of NADH was assayed spectrophotometrically by recording a decrease in the concentration of oxidized FC (ε_{420} = 1220 $M^{-1}\cdot cm^{-1}$) in the presence of saturating concentrations of the substrates: 0.8 mM NADH and 1.0 mM FC.

In all cases, the hydrogenase activities were determined in 0.1 M potassium phosphate buffer (pH 7.9) at 20°C.

Modification of His residues of the hydrogenase. All modifications of the hydrogenase were performed at 20°C in 0.1 M potassium phosphate buffer (pH 6.0-7.9).

The initial solution of DPC (5-10 mM) was prepared in absolute methanol. The precise concentration of DPC was determined spectrophotometrically at 242 nm in the presence of 0.025 M His (0.1 M potassium phosphate buffer, pH 7.9) using the molar extinction coefficient ε_{242} = 3200 $M^{-1}\cdot cm^{-1}$ for N-carboethoxyhistidine produced [19].

Modification of the nonactivated enzyme. To determine the rate of the hydrogenase modification, 1 ml of the enzyme solution was incubated in the cuvette section of a spectrophotometer (with a reference solution of the same composition but without the enzyme) in the presence of varied concentrations of DPC (0.17-1.0 mM). The modification of histidine residues was followed by changes in A_{242} with time that allowed us to plot the kinetic dependence of the number of modified His residues calculated per molecule of the protein. At certain time intervals, samples were taken from the reaction mixture to determine the enzyme activities in the reactions NADH + FC, H_2 + FC, and H_2 + NAD.

Modification of the activated enzyme under anaerobic conditions. One milliliter of the enzyme solution was placed into an "anaerobic" spectrophotometric cuvette, the cuvette was closed with a special rubber plug, and H_2 was flushed through the cuvette for 1-1.5 h. Then NADH solution, which was also flushed with hydrogen (the final concentration of NADH was 210 μM) was added, a sample was taken, the activity of the activated hydrogenase was determined, and then DPC solution was added. Kinetics of changes in A_{242} was recorded. At certain time intervals samples were taken from the reaction mixture for determination of the enzyme activities in the reactions of FC + NADH and H_2 + NAD. The reagents were added and samples were taken anaerobically using a gas-tight syringe.

In the control experiment, the activated hydrogenase was stable for at least 1 h.

Modification of thiol groups of the hydrogenase. In our work SH-groups of the hydrogenase from *R. eutropha* were modified with two thiol-modifying agents: DTNB and IAA. On treatment of the enzyme with DTNB the modification degree and changes in the activities of hydrogenase were recorded; on treatment with IAA only changes in the activities were recorded. The rate of the hydrogenase modification with DTNB was monitored spectrophotometrically by changes in the concentration of 5-thio-2-nitrobenzoate produced (ε_{412} = 13,600 $M^{-1}\cdot cm^{-1}$) [20].

Modification of the nonactivated enzyme with DTNB. Initial solutions of DTNB with the concentration of 6-10 mM were prepared in 0.1 M potassium phosphate buffer (pH 7.9). To determine the modification rate, 1 ml of the enzyme solution was incubated in the cuvette section of a spectrophotometer (with a reference solution of the same composition but without the enzyme) in the presence of varied concentrations of DTNB. Time-dependent changes in the A_{412} value were recorded. At certain time intervals samples were taken from the reaction mixture to determine the enzyme activities in the reactions FC + NADH and H_2 + NAD.

Modification with IAA. The initial solution of IAA with concentration of 100 mM was prepared in 0.1 M potassium phosphate buffer (pH 7.9).

Modification of the nonactivated enzyme. One milliliter of the enzyme solution with the concentration of 2-5 μM was placed into a penicillin bottle and IAA was added to the concentration of 10-12 mM. At certain time intervals samples were taken from the reaction mixture to determine the activities in the reactions FC + NADH and H_2 + NAD.

Modification of the activated enzyme under anaerobic conditions. One milliliter of the enzyme solution was placed into a penicillin bottle, the bottle was closed with a rubber plug, and hydrogen or argon was blown through for 1-1.5 h. Then NADH solution was added to the final concentration of 140-200 μM, a sample was taken, and the activity of the activated hydrogenase was determined; after that solution of IAA was added to the final concentration of 10-12 mM. Samples were taken from the reaction mixture to determine the hydrogenase and diaphorase activities. The reagents were added and samples were taken anaerobically with a gas-tight syringe. The solutions of NADH and IAA were previously flushed with hydrogen or argon.

RESULTS AND DISCUSSION

Modification of His residues of the NAD-dependent hydrogenase from *R. eutropha*. His residues of the NAD-dependent hydrogenase from *R. eutropha* were modified with DPC. This reagent is known to selectively interact with His residues at pH 6-8 with production of N-carboethoxyhistidine [19].

It has been shown [19, 21] that DPC interacts only with deprotonated imidazole His; therefore, the reaction rate increases with increase in pH value up to 8.0 (the p*K* of His imidazole is 6.8-7.0). On the other hand, DPC is hydrolyzed in aqueous solutions. The rate of hydrolysis increases with increase in pH [19].

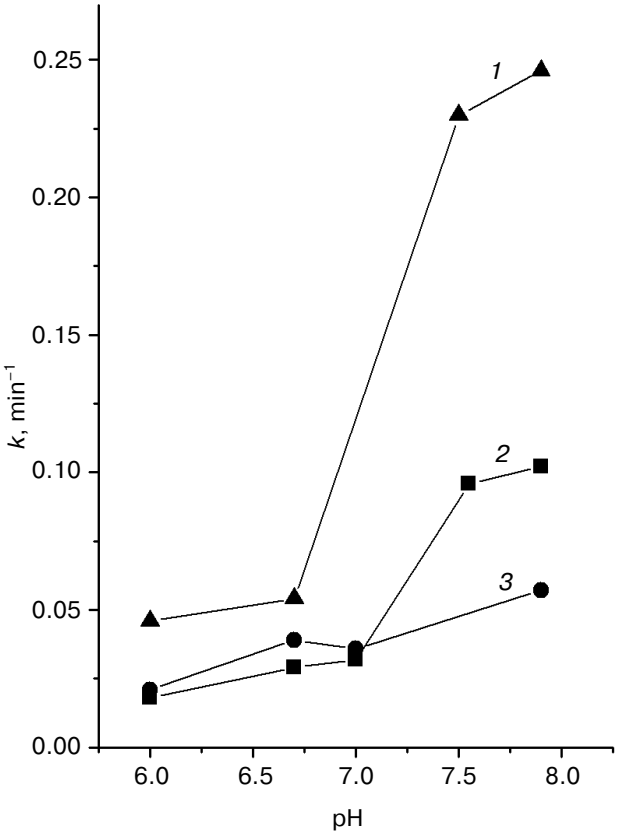

Fig. 1. pH dependence of rate constants of modification (*1*) and inactivation (the reaction FC + NADH) (*2*) of the hydrogenase in the presence of DPC, and also of the rate of DPC hydrolysis (*3*). Conditions: $[DPC]_0 = 0.44$ mM, 0.1 M potassium phosphate buffer, 20°C.

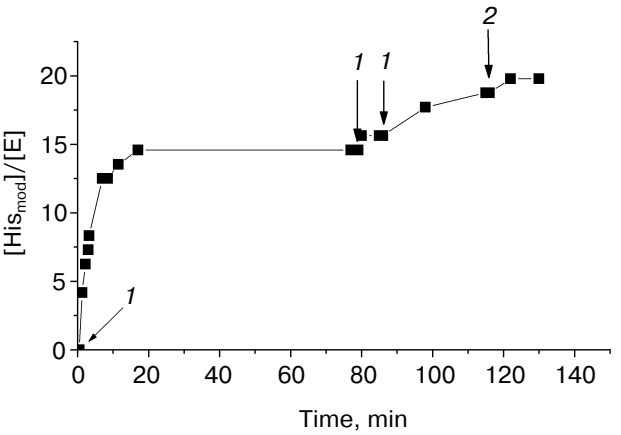

Fig. 2. Kinetics of modification of His residues of the hydrogenase under the influence of DPC. Arrows indicate the introduction of additional amounts of the modifying agent at the concentration of 0.3 (*1*) and 0.6 mM (*2*). Conditions: $[E]_0 = 1$ μM, 0.1 M potassium phosphate buffer, pH 7.9, 20°C.

To choose optimum conditions for the modification, pH-dependences of the rate of DPC hydrolysis and the rate of modification of hydrogenase in the presence of DPC were determined in the pH range from 6 to 8. As expected, the hydrolysis of DPC was described by first-order kinetics. The rate constant of the hydrolysis linearly depended on pH and increased 2.5-3-fold in the pH range from 6 to 8 (Fig. 1).

Rate constants of the hydrogenase modification were calculated by linearization of initial part of the kinetic curves of the modification (Fig. 2) by the method of Guggenheim [22] (Fig. 3). The linearization with the correlation coefficient of ~0.98 suggested that in the initial stages the modification was adequately described by pseudo first-order kinetics (the reaction was performed in the presence of 200-500-fold excess of the modifying agent). Deviation of the process from first-order kinetics at the later stages was associated with both decrease in the DPC concentration because of hydrolysis and decrease in the reactivity of the protein groups interacting with DPC. Figure 2 shows that introduction of additional amounts of

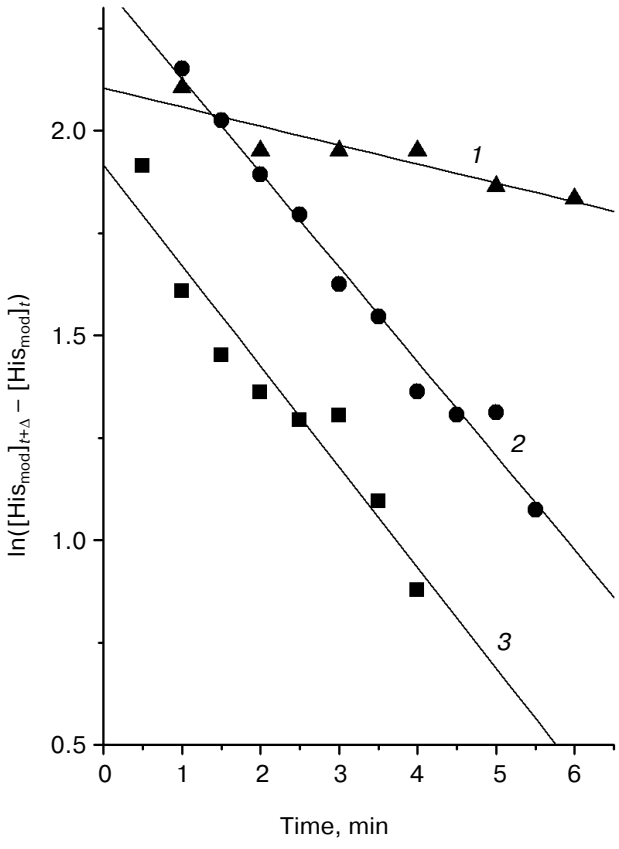

Fig. 3. Initial regions of kinetic curves of the hydrogenase modification with DPC at varied values of pH: 6.1 (*1*), 7.5 (*2*), 7.9 (*3*). Procession in semilogarithmic coordinates by Guggenheim's method [22]: $[His_{mod}]$ is the concentration of modified His residues at times t and $t + \Delta$, where Δ is 0.5 and 1 min.

DPC resulted in the further modification of the enzyme, but the rate of the process was noticeably lower.

The rate constants of the modification calculated as described above increased fivefold in the pH range from 6 to 8, and the dependence was not linear but rather S-like (Fig. 1). Because in the pH range under consideration the modification rate increased with increase in pH faster than the rate of DPC hydrolysis, the further modification was performed at pH 7.9.

The modification of His residues of the hydrogenase was associated with decrease in both hydrogenase and diaphorase activities of the enzyme (Figs. 4 and 5). The rate of the modification depended on the concentration of DPC. Linearization of initial regions of kinetic curves of the inactivation in semilogarithmic scale $\ln A_{res}$ on time t where the residual activity A_{res} was equal to the activity ratio at times t and 0 allowed us to assess the inactivation rates which were the same for all activities measured and, with regard to the DPC concentration, were 3.7-4.7 $M^{-1}\cdot sec^{-1}$ (the table). Because the hydrogenase and diaphorase activities in the reactions NADH + FC and H_2 + FC were located on different heterodimers [4], the equality of the inactivation rates suggested that catalytically significant His residues with similar reactivity should exist on both heterodimers. This assumption allowed us to explain why the values obtained for the inactivation rate constants were twofold lower than the modification rate constant (the table): this was due to recording the total modification rate of His residues on both heteromers.

Similarly to the modification rate, the rate of inactivation increased with increase in pH, and the rates of both processes changed virtually in parallel (Fig. 1). The pH dependence of the inactivation was S-like with the inflection point at about 7.0-7.5 that corresponded to the pK_a value of imidazole of His. Note that the inflection points of the left branches of the pH dependences of the maximum rates of both hydrogenase and diaphorase activities were also in the pH range of 6.9-7.5 [23]. It was suggested that just the deprotonation of the imidazole group of His should determine the possibility of acquisition by the enzyme of the maximum activity at the pH optimum of 7.8-8.0. That confirmed the significance of His residues for the functioning of both heterodimers of the hydrogenase.

A similar decrease in the catalytic activity under the influence of His-modifying agents was earlier shown for hydrogenase from *D. gigas* [24]. Modification of the first two His residues most significantly affected the catalytic activity. The authors of [24] assumed that besides His185 (the terminal element of the electron transfer chain locat-

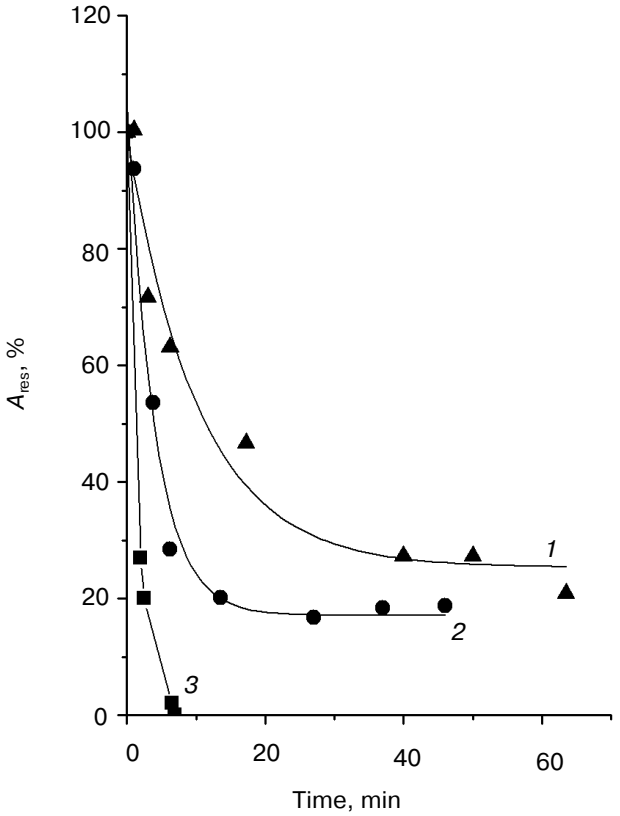

Fig. 4. Kinetics of inactivation by DPC of the hydrogenase activity of the activated (*3*) and nonactivated (*1*, *2*) hydrogenase (reaction H_2 + NAD). Conditions: $[E]_0$ = 1 μM, 0.1 M potassium phosphate buffer, pH 7.9, 20°C; $[DPC]_0$ = 0.37 (*1*), 0.74 (*2*), 0.44 mM (*3*).

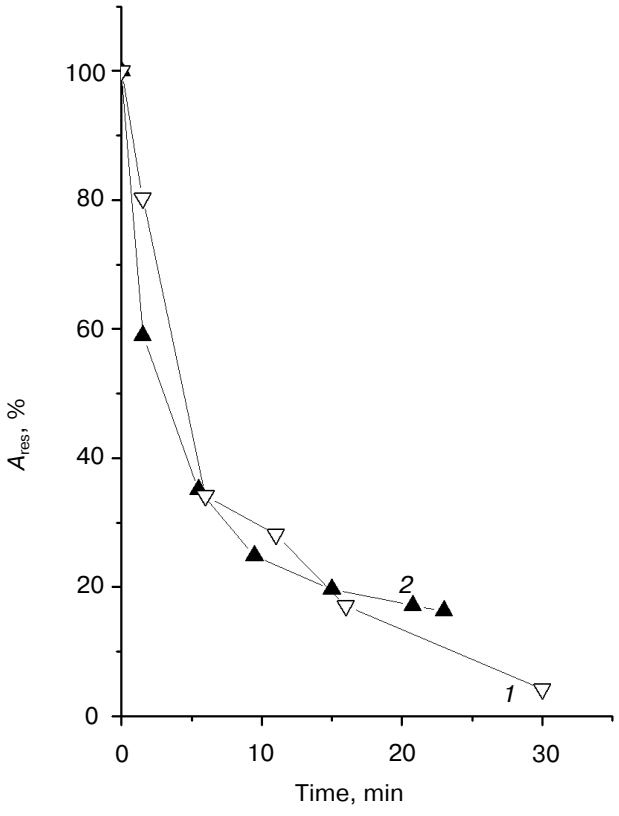

Fig. 5. Kinetics of inactivation by DPC of the diaphorase activity of the activated (*1*) and nonactivated (*2*) hydrogenase (reaction FC + NADH). Conditions: $[E]_0$ = 1 μM; $[DPC]_0$ = 0.38 (*1*), 0.41 mM (*2*); 0.1 M potassium phosphate buffer, pH 7.9, 20°C.

Rate constants of the modification (k_{mod}) and inactivation (k_{in}) of the hydrogenase from *R. eutropha* under the influence of diethylpyrocarbonate (DPC)

Activity type	Enzyme state during the modification	[DPC], mM	$k_{mod}(k_{in})$, min^{-1}	$k = k_{mod}(k_{in})/[DPC]$, $M^{-1}\cdot sec^{-1}$	k_{av}, $M^{-1}\cdot sec^{-1}$
		Modification			
	nonactivated	0.42	0.25	9.8	9.8
	activated	0.55	1.98	60	60
		Inactivation			
Diaphorase NADH + FC	nonactivated	0.17 0.44 0.86	0.038 0.157 0.23	3.7 5.9 4.6	4.7 ± 1.2
	activated	0.38	0.1	4.4	4.4
Hydrogenase H_2 + NAD	nonactivated	0.37 0.74 1.44	0.08 0.20 0.26	3.6 4.5 3.0	3.7 ± 0.8
	activated	0.44	0.60	22	22
Hydrogenase H_2 + FC	nonactivated	0.24 0.47	0.08 0.11	5.5 3.9	4.7 ± 0.8

ed on the protein surface), the His residue in the proton-removing channel was modified. Such a modification was not associated with changes in the structure of the hydrogen-activating site because the rate of *ortho/para* conversion of hydrogen located at the active site was not changed after the modification. But the rates of isotope exchange and reduction of methyl viologen with molecular hydrogen, which are accompanied by transport of protons, decreased.

All above-presented data pertain to the modification of the nonactivated (as prepared) enzyme. Similarly to other hydrogenases, the hydrogenase from *R. eutropha* isolated in the presence of oxygen (in air) is inactive and requires preactivation under reducing conditions (H_2, NADH, dithionite) [1-4, 25, 26].

On activation of the hydrogenase under anaerobic conditions (H_2 + NADH) and its subsequent treatment with DPC, the modification rate of His residues increased approximately sixfold. The inactivation rate of the hydrogenase activity also increased five-sixfold (Fig. 4, the table). The rate of loss of the diaphorase activity was virtually the same as in the case of the nonactivated enzyme (Fig. 5, the table). In the absence of DPC, the dehydrogenase was stable under these conditions within at least half an hour, whereas the half-inactivation period of the diaphorase activity in the presence of DPC was 6-7 min and that of the hydrogenase activity was about 1.5-2 min.

The findings suggested that a functionally significant His residue (or a number of such residues) existed inside the protein molecule, and this residue was available for a modifying agent only on the enzyme activation with NADH under anaerobic conditions. Because the modification of this residue sharply increased the rate of inactivation of the hydrogenase activity at the constant rate of decrease in the diaphorase activity, it was suggested that this residue should be located on the hydrogenase heterodimer. Activation of the hydrogenase seemed to result in availability for the modification of His residues of the proton channel located inside the protein molecule, in particular, His69, located immediately near the [Ni-Fe]-site and homologous to His72 in *D. gigas* [15, 16].

Thus, the activation in the presence of NADH and H_2 resulted in significant structural rearrangements in the active site of the NAD-reducing hydrogenase, and this supported the earlier data that the activation was associated with changes in the enzyme resistance to various inactivating agents (urea, temperature, limited proteolysis) [25] and also data of X-ray absorption spectroscopy on the structure of reduced and oxidized forms of the enzyme [26].

These conclusions are also supported by results of the modification of thiol groups in the hydrogenase from *R. eutropha*.

Modification of thiol groups of the NAD-dependent hydrogenase from *R. eutropha*. Notwithstanding the important role of thiol groups in the functioning of hydrogenases, the treatment of a number of nonactivated Ni-Fe hydrogenases with reagents interacting with thiol

groups failed to significantly inactivate these enzymes [27-30]. It is usually explained by unavailability of significant thiol groups for modifying agents due to location of Cys clusters and prosthetic groups coordinated by them in the hydrophobic cavity inside the protein globule. Activation of hydrogenases increases their sensitivity to thiol-modifying agents [29, 30], and increase in the modification degree of thiol groups on the activation is first of all associated with modification of additional Cys residues on the subunit which includes the hydrogen-activating site [30] (the case at hand is about dimeric Ni-Fe hydrogenases which correspond to the HoxHY-dimer of the enzyme from *R. eutropha*).

In our work, thiol groups of the hydrogenase were modified with two specific reagents, DTNB and IAA. On treatment of the nonactivated enzyme with DTNB, eight or nine SH-groups were modified per protein molecule at the molar ratio of [DTNB]/[hydrogenase] equal to 30. The further increase in the DTNB concentration insignificantly increased the modification degree (to 10.5 SH-groups per protein molecule at the [DTNB]/[hydrogenase] ratio of about 5000) (Fig. 6). According to work [2], the total number of SH-groups in a molecule of the hydrogenase from *R. eutropha* is 41. The modification was not described by first-order kinetics, but was a superposition of reactions going with different rates because of different reactivity and availability of thiol groups of the protein. The initial rate of the protein modification at the [DTNB]/[hydrogenase] ratio equal to 30 was $5 \cdot 10^{-3}$ sec^{-1} and approximately the same as the rate of the hydrogenase modification ($6 \cdot 10^{-3}$ sec^{-1}) at the ratio of DTNB to the enzyme concentration equal to 1.5 : 1, when the final modification degree was one SH-group per protein molecule. It seems that this was the modification rate of the most reactive group of the nonactivated enzyme.

The modification under the influence of DTNB of eight to ten SH-groups per molecule of the nonactivated hydrogenase did not significantly change the activity of the enzyme (Fig. 6).

Treatment of the nonactivated hydrogenase with another modifying agent, IAA, also was not accompanied by changes in the hydrogenase and diaphorase activities for 4 h. The modification degree in this case was no lower than in the case with DTNB, because the addition 2 h later of 10 mM DTNB into the reaction mixture containing the enzyme and IAA failed to cause an additional modification of thiol groups, i.e., to increase the value of A_{412}.

Treatment with iodoacetamide of the hydrogenase activated by NADH in the atmosphere of argon resulted in a rapid decrease in the hydrogenase activity. The diaphorase activity was relatively stable (Fig. 7, curves *1* and *2*).

The modification with iodoacetamide of the hydrogenase activated by NADH in the atmosphere of hydrogen resulted in the loss of both the hydrogenase and diaphorase activities (Fig. 7, curves *3* and *4*). All kinetic curves of the inactivation of the activated enzyme were not described by the first-order equation but were produced by superposition of a number of processes with different rates. Rapid initial regions of the kinetic curves of

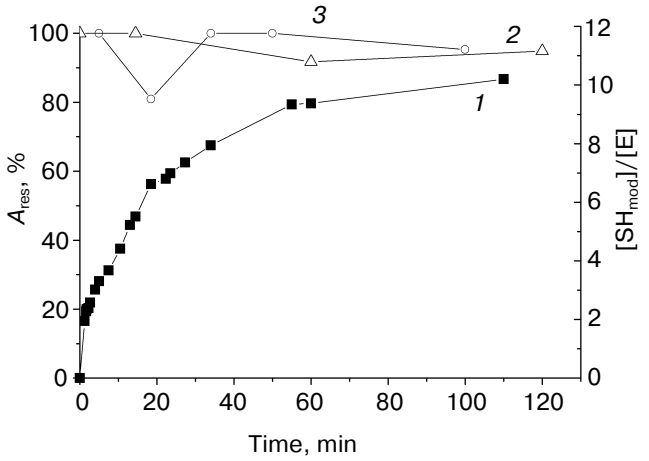

Fig. 6. Kinetics of modification (*1*) and changes in the diaphorase (*2*) and hydrogenase (*3*) activities on treatment of the nonactivated hydrogenase with DTNB. Conditions: $[E]_0$ = 2.0 μM; 0.1 M potassium phosphate buffer, pH 7.9, 20°C; [DTNB] = 10 mM.

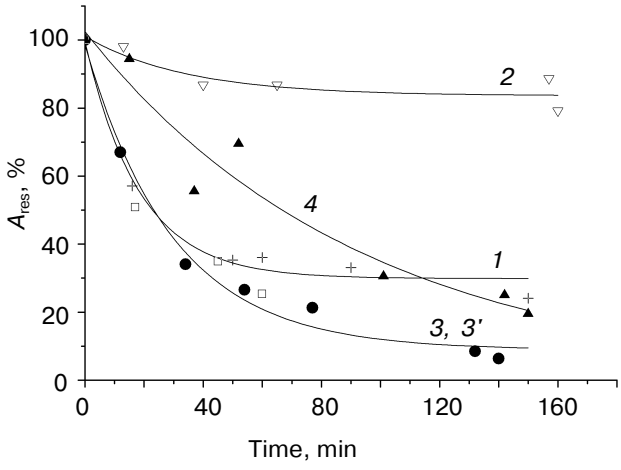

Fig. 7. Kinetics of inactivation of the hydrogenase (*1*) and diaphorase (*2*) activities during the modification with IAA of the hydrogenase activated with NADH in an atmosphere of argon. The NADH concentration during the activation was 0.14 mM. Kinetics of inactivation of the hydrogenase (*3* and *3′*) and diaphorase (*4*) activities at the modification with IAA of the hydrogenase activated with NADH in the atmosphere of hydrogen. The NADH concentration during the activation was 1.4 (*3′*) and 0.15 mM (*3*, *4*). Conditions: $[E]_0$ = 2.4 μM; 0.1 M potassium phosphate buffer, pH 7.9, 20°C; [IAA] = 10 mM.

the loss of the hydrogenase activities coincided for the enzymes activated by NADH in the atmosphere of hydrogen and argon (Fig. 7, curves *3* and *1*). They seemed to be associated with the modification of thiol groups on the HoxH-subunit involved in the coordination of the active [Ni-Fe]-site, which became available in the activated enzyme. However, the inactivation of the hydrogenase activated by NADH in the atmosphere of hydrogen did not stop at this stage but continued to deeper transformations via slow processes. It is possible that slow stages of inactivation were associated with the modification of Cys residues involved in the coordination of Fe-S-clusters, as suggested in [27]. The loss of the diaphorase activity on the modification of the activated enzyme seemed to be due to the same cause (Fig. 7, curve *4*). The findings suggested that the hydrogenase activation by NADH in the atmosphere of hydrogen should be associated with deeper structural rearrangements than on other modes of the activation, and this is in agreement with the earlier finding [31] that the hydrogenase activation by NADH in the atmosphere of hydrogen resulted in the more active but less stable enzyme than the activation by NADH in an atmosphere of nitrogen.

The findings confirm the functional significance of Cys residues for both the hydrogenase and diaphorase fragments. The modification of SH-groups of the NADH-activated hydrogenase significantly inactivates the enzyme, whereas it remains virtually completely resistant to modifiers in the nonactivated state. The modification degree and changes in the corresponding catalytic activities depend on conditions of the enzyme activation.

Experiments on modification of both cysteine and histidine residues of the hydrogenase suggest significant conformational changes in the protein globule as a result of enzyme activation.

This work was supported by the Russian Foundation for Basic Research (project No. 98-04-48871).

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