Cysteine reactivity in *Thermoanaerobacter brockii* alcohol dehydrogenase

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Abstract

The free cysteine residues in the extremely thermophilic *Thermoanaerobacter brockii* alcohol dehydrogenase (TBADH) were characterized using selective chemical modification with the stable nitroxyl biradical bis(1-oxy-2,2,5,5-tetramethyl-3-imidazoline-4-yl)disulfide, via a thiol–disulfide exchange reaction and with 2[¹⁴C]iodoacetic acid, via S-alkylation. The respective reactions were monitored by electron paramagnetic resonance (EPR) and by the incorporation of the radioactive label. In native TBADH, the rapid modification of one cysteine residue per subunit by the biradical and the concomitant loss of catalytic activity was reversed by DTT. NADP protected the enzyme from both modification and inactivation by the biradical. RPLC fingerprint analysis of reduced and S-carboxymethylated lysyl peptides from the radioactive alkylated enzyme identified Cys 203 as the readily modified residue. A second cysteine residue was rapidly modified with both modification reagents when the catalytic zinc was removed from the enzyme by *o*-phenanthroline. This cysteine residue, which could serve as a putative ligand to the active-site zinc atom, was identified as Cys 37 in RPLC. The EPR data suggested a distance of ≤ 10 Å between Cys 37 and Cys 203. Although Cys 283 and Cys 295 were buried within the protein core and were not accessible for chemical modification, the two residues were oxidized to cystine when TBADH was heated at 75 °C, forming a disulfide bridge that was not present in the native enzyme, without affecting either enzymatic activity or thermal stability. The status of these cysteine residues was verified by site directed mutagenesis.

Keywords: alcohol dehydrogenase; carboxymethylation; chemical modification; cysteine; EPR; nitroxyl biradical; thiol–disulfide exchange

The thiol groups of cysteines are important for protein structure and folding (Creighton, 1992). As in other proteins, chemical modification of reactive cysteine residues has been used extensively to investigate the importance of cysteine residues in the structure and function of the alcohol dehydrogenases (ADHs). In zinc-containing ADHs, cysteine serves as a ligand for the catalytic zinc atom (Brändén et al., 1973). In most eukaryotic and in some prokaryotic ADHs, cysteine serves as a ligand for a structural zinc atom as well (Vallee & Auld, 1990). The role of other cysteine residues that are present in the ADHs has not yet been determined.

Of the three catalytic zinc ligands that have been identified in the 3D structure of horse liver ADH (HLADH), the only enzyme for which a detailed 3D structure of an ADH with its coenzyme is currently available (Brändén et al., 1973), two residues, Cys 46 and Cys 174, can be selectively alkylated by α -halo acids (Reynolds & McKinley, 1969; Zeppezauer et al., 1975; Dahl & McKinley, 1981; Chadha & Plapp, 1984; Johansson et al., 1991) in an affinity-labeling type of reaction (Li & Vallee, 1963; Harris, 1964). Whereas carboxymethylating Cys 46 and Cys 174 of HLADH, or the putative active site zinc ligand cysteines of human ADH (Johansson et al., 1991), sheep liver sorbitol dehydrogenase, and human liver aldehyde dehydrogenase (Johansson et al., 1992), abrogates enzymatic activity, such modification of the ADH from *Sulfolobus sulfataricus* significantly increases the oxidation rate of alcohols (Raia et al., 1996).

Thermoanaerobacter brockii is a hyperthermophilic, anaerobic bacterium that was originally isolated from the Yellowstone Park hot springs (Lamed & Zeikus, 1981). This microorganism contains an NADP-linked secondary ADH (TBADH) that exhibits extreme thermostability ($T_m = 93^\circ$) (Bogin et al., 1996). TBADH has been purified in our laboratory and characterized as a homotetramer composed of 352 amino acid residues (Peretz & Burstein, 1989).

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Abbreviations: ADH, alcohol dehydrogenase; HLADH, horse liver ADH; TBADH, *Thermoanaerobacter brockii* alcohol dehydrogenase; CM, carboxymethyl; DTT, *d,l* dithiothreitol; Gdn-HCl, guanidine hydrochloride; nitroxyl radical, 1-oxy-2,2,5,5-tetramethyl-3-imidazoline-4-yl)sulfide; nitroxyl biradical, bis(1-oxy-2,2,5,5-tetramethyl-3-imidazoline-4-yl)disulfide; R⁺Hg-Cl, 2,2,5,5-tetramethyl-4-(2-(chloromercuri)phenyl)-3-imidazoline-1-oxyl; TEMPO, 2,2,6,6-tetramethyl-1-piperidinyloxy; EPR, electron paramagnetic resonance; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

and one zinc atom per subunit (Bogin et al., 1997). The primary structure of TBADH contains four cysteine residues at positions 37, 203, 283, and 295 (Peretz & Burstein, 1989), but the reactivity of these residues has not yet been determined. Aligning the amino acid sequence of TBADH with those of ADHs from other organisms (Sun & Plapp, 1992) has shown that of the four cysteines in TBADH, the only cysteine residue that is preserved in all the zinc-containing ADHs is Cys 37, which corresponds to the HLADH catalytic zinc ligand Cys 46 (Brändén et al., 1973).

In this report, we describe the use of selective chemical modification to study the properties of the cysteine residues of TBADH. To modify the cysteine residues, we used S-carboxymethylation with [^{14}C]iodoacetic acid. We also used a thiol-disulfide exchange reaction, using a stable nitroxide biradical in which two nitroxide groups are linked by a disulfide bond (Khramtsov et al., 1989; Dolginova et al., 1992; Weiner, 1995), as described in the following equation:

where HS-E is a free thiol group of the protein, and R^* is the nitroxyl radical. The modification reaction (Equation 1) leads to the formation of two monoradicals. One radical is covalently bound to the protein ($\text{R}^*\text{S-S-E}$) and allows traditional spin-label measurement, which is related to such properties as protein mobility, environment, interaction with substrates and inhibitors, and so on (Berliner, 1976; Berliner & Reuben, 1989). The other monoradical ($\text{R}^*\text{-SH}$), released into the solution during the course of the reaction, enables direct measurement of the rate of the chemical modification, which reflects on the structural and chemical properties of the modified sulfhydryl group (Weiner, 1995).

Results

Kinetics of the chemical modification and inactivation of TBADH by the $\text{R}^*\text{S-SR}^*$ biradical

The results presented in Figure 1 indicate that the $\text{R}^*\text{S-SR}^*$ biradical is an effective inhibitor of TBADH. The kinetic parameters of inactivation were calculated according to an approach suggested previously (Levy et al., 1963). The biradical inhibited enzymatic activity with pseudo-first-order kinetics. The reaction order with respect to biradical concentration (Fig. 1, inset) was 0.96 ± 0.11 , which is consistent with inactivation by 1 mol of radical per subunit of TBADH. Figure 2 shows that NADP protected the enzyme from both modification and inactivation by the biradical, and that DTT restored the activity of the modified inactive enzyme.

Electron paramagnetic resonance (EPR) spectroscopy study of the modification

We used electron paramagnetic resonance (EPR) spectroscopy to study the availability of the cysteine residues of TBADH for chemical modification with the biradical. The rate of the thiol exchange reaction was followed by monitoring the increase in the EPR signal of the monoradical released into the solution during the course of the reaction, according to Equation 1. Figure 3A shows the increase in the EPR signal for the reaction between the biradical and native TBADH in the absence of NADP. In the presence of the cofactor (Fig. 3C), the rate of monoradical production was considerably slower. Apo-TBADH (<0.05 g atom zinc per subunit)

Fig. 1. Kinetic of inactivation of TBADH by the nitroxyl biradical. TBADH (10 μM) was incubated with biradical concentrations of 0.5 mM (open squares), 1 mM (solid squares), 2 mM (open triangles), and 4 mM (open circles) in 50 mM Tris-HCl (pH = 7.4) at 37 $^{\circ}\text{C}$, and the residual enzymatic activity was monitored as a function of time. Insert: double logarithmic plot of the rate constants versus the concentration of the biradical.

(Fig. 3B) reacted with the biradical at about twice the initial rate of that of native TBADH, whereas an alkyl-mercury derivative of TBADH (see below) was modified very slowly at $<5\%$ of the initial rate of that of native TBADH (Fig. 3D).

Fig. 2. The effect of NADP and DTT on the inhibition of TBADH by the biradical. TBADH (10 μM) in 50 mM Tris-HCl (pH = 7.4) was incubated with 2 mM biradical without (closed circles) and with (open circles) 5 mM NADP, and the residual enzymatic activity (percent) was monitored as a function of time. DTT (5 mM) was introduced at 10 min (shown by arrow).

Fig. 3. Kinetics of the nitroxyl biradical modification of TBADH. The peak intensity of the EPR spectrum of the monoradical component released to solution as a result of the thiol–disulfide exchange between TBADH and the biradical (Eq. 1) was plotted as a function of time. The enzyme preparation ($4\ \mu\text{M}$) was added to a solution of the biradical ($20\ \mu\text{M}$) in $10\ \text{mM}$ Tris-HCl ($\text{pH} = 7.4$). (A) Native TBADH; (B) apo-TBADH (<0.05 g atom zinc/subunit); (C) TBADH with NADP ($5\ \text{mM}$); (D) TBADH pretreated with methylmercury acetate.

EPR labeling of TBADH

Figure 4 shows the EPR spectrum of TBADH modified by the biradical (after removing the excess reagents by gel filtration). Such a spectrum ($2A_{zz} = 64.1\ \text{G}$) is typical for an immobilized radical (Morrisett, 1976). An EPR signal corresponding to a small amount of unbound monoradical ($<5\%$) can be detected. The stoichiometry calculated for the immobilized radical ($n = 1.83 \pm 0.2$ per subunit) indicates that two thiol groups per subunit were modified in TBADH. Note that although the rate of modification of the holoenzyme (Fig. 3A) was only about half that of the modification of the apo-enzyme (Fig. 3B), both proteins were eventually modified to the same extent (under somewhat different reaction conditions, see Discussion and Materials and methods). Atomic absorption spectroscopic analysis showed that the holoenzyme, modified by $\text{R}^*\text{S-SR}^*$ (Fig. 4), lost $>90\%$ of its zinc upon modification.

Adding excess DTT to the modified enzyme transformed the EPR spectrum (Fig. 4B). The broad peaks corresponding to the covalently bound spin-label disappeared completely, and the magnitude of the sharp peaks that are characteristic of unbound, free-rotating radicals in solution increased.

Effect of temperature on the EPR spectrum of modified TBADH

Figure 5 shows the effect of temperature on the EPR spectrum of TBADH modified by Hg-R^* . At low temperature ($298\ \text{K}$, Fig. 5A), a wide peak that corresponds to the immobilized radical appeared at low field. At high temperature ($353\ \text{K}$, Fig. 5B), the peak narrowed and shifted to high field. As the intensity of this peak

decreased (Fig. 5), that of the EPR signal attributed to a *non-immobilized* radical tended to increase. The EPR spectra at high temperature were characterized by two additional peaks that were located between the signals of a free rotating radical. The signals at this site are typical of stable nitroxyl biradicals, characterized by a strong exchange interaction between spins of *unpaired* electrons ($J > a$, where J is the exchange integral and a is the hyperfine constant) (Luckhurst, 1976).

Carboxymethylation of free cysteines in TBADH and in apo-TBADH

EPR measurements showed that only two cysteines of TBADH were available for reversible chemical modification with the biradical. One cysteine residue was protected by NADP and could be modified only in its absence, while the other cysteine residue could be readily modified in the absence of the catalytic zinc. To identify these residues in the TBADH molecule, we repeated the modifications using a more stable and radioactively labeled derivative.

Titration of the free sulfhydryl groups of TBADH with $[2\text{-}^{14}\text{C}]\text{iodoacetic acid}$ under non-denaturing conditions, both in the presence of and in the absence of DTT ($10\ \text{mM}$), resulted in the incorporation of about one carboxymethyl group per subunit ($1\text{CM}^*\text{-TBADH}$, Table 1). $1\text{CM}^*\text{-TBADH}$ was devoid of enzymatic activity. NADP prevented the modification (Table 1) and enzymatic activity was preserved. Titration of apo-TBADH with $[2\text{-}^{14}\text{C}]\text{iodoacetic acid}$ led to the incorporation of about two carboxymethyl groups into the enzyme ($2\text{CM}^*\text{-TBADH}$, Table 1).

Fig. 4. EPR Spectrum of the TBADH-biradical conjugate. The conjugate (80 μ M, prepared as described in Materials and methods) was measured in 50 mM Tris-HCl (pH = 7.4). EPR conditions: (A) microwave power 20 mW, modulation amplitude 1 G, gain 2×10^4 ; (B) EPR spectrum of the conjugate after the addition of 1 mM DTT: gain 10^3 ; (C) EPR spectrum of sample B at gain 2×10^4 (as in A).

Identification of the alkylated cysteine(s) in TBADH and apo-TBADH

To identify the [2- 14 C]carboxymethylated cysteine residues of 1CM*-TBADH and 2CM*-TBADH, the modified enzymes were first reduced with excess DTT in 6 M Gdn-HCl and carboxymeth-

ylated with [12 C]iodoacetate; the carboxymethylated enzymes were then digested with API in 2 M urea. The peptide digest was separated by RPLC as described previously (Peretz & Burstein, 1989). All four CM-cysteine containing peptides that are known to be present in peptide peaks K5 (Cys 203), K12 (Cys 283), K14 (Cys 37), and K15 (Cys 295) (Peretz & Burstein, 1989) were well separated, as shown in Figure 6.

When 1CM*-TBADH was subjected to such treatment, only one major (>80%) radioactive peptide peak, K5, was present (data not shown), indicating that in the native enzyme only Cys 203 was readily available for alkylation with iodoacetate.

2CM*-TBADH yielded two radioactive peptide peaks, K5 and K14 (Fig. 6). In this modification, both Cys 203 and Cys 37 were alkylated. Peptides K5 and K14 were isolated and subjected to amino acid composition analysis. The results confirmed the expected composition of the lysyl peptides (Peretz & Burstein, 1989) and showed that the only carboxymethylated amino acid was cysteine (data not shown).

The buried cysteines of TBADH

Under mild conditions, in either the presence or the absence of DTT, the remaining two cysteine residues of TBADH, Cys 283 and Cys 295, could not be modified. S-Carboxymethylation of these residues could be achieved only after denaturing the protein with 6 M Gdn-HCl. Indeed, when 2CM-TBADH was prepared (see above) using [12 C]iodoacetic acid and then subjected to carboxymethylation with [2- 14 C]iodoacetic acid in 6 M Gdn-HCl, about two [2- 14 C]carboxymethyl groups were incorporated into 2CM-TBADH to form 4CM*-TBADH (radioactively labeled, Table 1). API digestion of 4CM*-TBADH followed by RP-HPLC separation produced two radioactive lysyl peptide peaks, K12 and K15 (Table 1), indicating that Cys 283 and Cys 295 became available for alkylation only after the enzyme had been completely denatured.

Heat-induced formation of a disulfide bridge between Cys 283 and Cys 295 in TBADH

In previous research (Peretz & Burstein, 1989), the original isolation procedure for TBADH included a step of heating at 70–75 $^{\circ}$ C for 30 min. TBADH isolated after such treatment was enzymatically active (up to 85 U/mg) (Peretz & Burstein, 1989) and thermostable ($T_{1/2}^{60\text{min}} = 93^{\circ}$ C) (Bogin et al., 1996). Only two cysteines, Cys 37 and Cys 203, were identified in such heat-treated preparations of TBADH (Peretz & Burstein, 1989). The other two cysteine residues could not be alkylated even under denaturing conditions, unless a disulfide reducing agent (DTT or 2-mercaptoethanol, for example) was included. In the present study, we digested the enzyme with trypsin to determine the status of the "missing" cysteine residues in heat-treated TBADH. Figure 7A shows the RPLC fingerprint profile of the tryptic peptides isolated from the digest. The peptides were isolated and characterized by protein sequence analysis and amino acid composition analysis, which in peptide T9 revealed the presence of two peptide sequences (Fig. 7A) having the following amino acid sequence (two or one amino acids in each sequence cycle): (Leu/Gly)-(Glu/Gly)-(Trp/Leu)-Gly-Pro-Gly-(Met/Gly)-(Ala/Arg)-His-Lys.

After peptide T9 was reduced and S-carboxymethylated, the RP-HPLC profile, under identical conditions, showed the presence of two distinct peptides, denoted T9a and T9b in Figure 7B. Amino acid sequence analysis of the isolated peptides indicated that peptide T9a contained residues 292–299 and peptide T9b contained

Fig. 5. EPR spectrum of TBADH modified by Hg-R* nitroxyl radical. The conjugate (10 μ M, prepared as described in Materials and methods) was measured in 25 mM Tris-HCl pH = 7.5. EPR conditions: microwave power 20 mW, modulation amplitude 1 G, gain 2×10^5 ; (A) at 298 K; (B) at 353 K (arrows indicate the positions of the new EPR components).

Table 1. [^{14}C]carboxymethylation of cysteine residues in TBADH

Modification of TBADH with reactants ^a	[^{14}C]CMCys ^b (mol/subunit) ^c			
	37	203	283	295
Gdn.HCl ^d + DTT + [^{14}C]IAA ^d	0.9	0.8	1.0	1.0
Gdn.HCl + [^{14}C]IAA	1.0	0.9	1.0	0.9
(apo-TBADH) ^e + [^{14}C]IAA	0.9	1.0	<0.05	<0.05
NADP + [^{14}C]IAA	0.3	0.2	<0.05	<0.05
[^{14}C]IAA	0.3	1.0	<0.05	<0.05
[^{12}C]IAA $\rightarrow$ ^f Gdn.HCl + DTT + [^{14}C]IAA	0.7	<0.05	1.0	0.9
[^{12}C]IAA $\rightarrow$ ^f [^{14}C]IAA	0.2	<0.05	<0.05	<0.05
CH ₃ -Hq-TBADH + [^{14}C]IAA	0.3	<0.05	<0.05	<0.05

^aTBADH (1 mg in 0.5 mL) was alkylated with iodoacetic acid (10–100 mM), for 30 min in 500 mM Tris-HCl, pH 8.0–8.5 in the dark. The reaction was stopped by adding excess DTT followed by exhaustive dialyzed against ammonium bicarbonate (50 mM) at 4°C for 24 h.

^bCMCys, S-carboxymethylcysteine.

^cAfter partial alkylation, the protein was exhaustively alkylated with [^{12}C]IAA in Gdn-HCl. The completely carboxymethylated protein was digested with API and peptide fingerprint analysis was performed (for details see text).

^dGdn-HCl, 6 M guanidine-HCl; IAA, 2-iodoacetic acid.

^eapo-TBADH, <0.05 g atom zinc per subunit.

^fNonradioactive alkylation for 30 min followed by radioactive alkylation.

residues 279–288 of the TBADH primary structure, suggesting that Cys 283 (T9a) and Cys 295 (T9b) in peptide T9 may have been oxidized to cystine, forming a disulfide bridge to link the two peptides, T9a and T9b.

*Further modification of Cys 203 of TBADH
by S-methyl-mercuration and
S-[^{14}C]cyanylation ([^{14}C]NTCB)*

The ability of NADP to prevent the iodoacetate alkylation of Cys 203 could have resulted either from steric hindrance or from

a direct interaction(s) between the sulfhydryl group of the enzyme and a functional group(s) of the co-enzyme. To examine these possibilities, we further modified Cys 203 of TBADH by S-methyl-mercuration and by S-[^{14}C]cyanylation. Methylmercury acetate inactivated TBADH, the mercurial derivative of the enzyme did not react with the biradical (Fig. 3D) or with iodoacetate (data not shown), and adding NADP prevented the modification of TBADH. [^{14}C]NTCB (Degani et al., 1970) modification of TBADH resulted in the incorporation of 1 mol of [^{14}C]thiocyanate and the loss of over 85% of the enzymatic activity. NADP prevented the modification, as before.

Fig. 6. RPLC fingerprint profile of an API digest of 4CM*-TBADH. [^{14}C]carboxymethylated TBADH (1–5 mg) was digested with *Achromobacter* protease I (see Materials and methods), and 10–30% of the digest was loaded onto an RPLC column (Aquapore RP-300, 4.6 × 20 mm). The column was eluted with a linear gradient of acetonitrile (5–55% in 0.1% aqueous trifluoroacetic acid) in 50 min at a flow rate of 1 mL/min.

Fig. 7. Isolation of a cysteine containing peptide from a tryptic digest of heat-oxidized TBADH. **A:** The tryptic peptide mixture (1–10 nmol) was fractionated by RPLC and monitored as described in the legend to Figure 6. Peptide T9 (cysteine) was characterized by protein sequence analysis. **B:** Peptide T9 was reduced with DTT in Gdn-HCl, carboxymethylated with iodoacetic acid (see Materials and methods), and the reaction mixture was rechromatographed as in A. Peptides T9a and T9b were characterized by amino acid composition analysis.

Site-directed mutagenesis of Cys 203 of TBADH

Using standard procedures (Sambrook et al., 1989), we constructed two mutants at the Cys 203 position, Cys 203-Ala-TBADH and Cys 203-Ser-TBADH. The expression and isolation of recombinant TBADH and its mutants was performed as described previously (Peretz et al., 1997). Whereas Cys 203-Ala-TBADH retained only 20% of the enzymatic activity of native TBADH, the enzymatic activity of Cys 203-Ser-TBADH was 2.5-fold higher than that of the native enzyme.

Site-directed mutagenesis of Cys 283 of TBADH

To examine whether the covalent disulfide bridge consisting of Cys 283–Cys 295 that was formed in heat-treated TBADH (see above) contributed to the thermostability of the cysteine-containing TBADH derivative, we replaced Cys 283 with serine by site-directed mutagenesis and isolated and characterized the mutant enzyme. Both the $T_{1/2}^{60\text{min}}$ value (93 °C) and the specific enzymatic activity of Cys-283-Ser-TBADH (82 U/mg) were nearly identical to those of the native enzyme.

Discussion

The kinetics of the chemical modification and inactivation of TBADH by the nitroxyl biradical indicated that in the absence of the coenzyme, one SH group of TBADH was readily available for modification and that a second cysteine could be modified at a much slower rate. That NADP prevented only the modification of the fast-rate reaction suggested that the readily modified cysteine might be located on the surface of the enzyme, near or at the binding site of the coenzyme. We could not rule out the possibility, however, that this cysteine residue was located elsewhere in the protein molecule and that an NADP-induced conformational change was responsible for the protection. Radioactive carboxymethylation of TBADH with [^{14}C]iodoacetic acid revealed Cys 203 as the site of the SH group that was readily available for alkylation. As this type of modification also inactivated the enzyme, was prevented by NADP, and both modifications were mutually excluded, the biradical modification presumably involved Cys 203.

One explanation for the concomitant inactivation of TBADH by modifying Cys 203 could be that the chemical modification caused a conformational change or prevented the correct binding of NADP to the modified enzyme, either by blocking part of the coenzyme binding site or by abolishing an essential bond between the coenzyme and the SH group of the enzyme, or both. The fact that the intrinsic fluorescence spectra and ANS (1-anilino-8-naphthalene-sulfonic acid) binding of non-modified and labeled TBADH derivatives were practically identical (data not shown) indicated that no gross conformational changes were caused by the modifications. It is, therefore, unlikely that loss of enzymatic activity was due to loss of structure.

Cysteine residues that are important to protein structure or that are in the active site, as well as those participating in catalytic or regulatory activities, are conserved in protein families. Residue 203 is not conserved in all ADHs (Sun & Plapp, 1992), and Cys 203 is present in very few ADHs, indicating that this residue does not play a crucial role in maintaining either the structure or the function of these enzymes. Aligning the amino acid sequences of TBADH and HLADH has shown that Cys 203 of TBADH is analogous to Lys 228 of HLADH (Peretz & Burstein, 1989). The side chain of Lys 228 points toward the surface of the HLADH molecule, in the region of the coenzyme binding site (Eklund et al., 1984). Assuming similar structures for the ADP-moiety of the coenzyme-binding sites of HLADH and TBADH, inactivation of the chemically modified enzyme could have resulted from steric hindrance by either the bulky -S- carboxymethyl or the even bulkier -S-nitroxyl-radical moieties. Indeed, when the side chain (R) of residue 203 was modified with moieties smaller than the monoradical ($\text{R} = -\text{CH}_2-\text{S}-\text{S}-\text{R}^*$) or the carboxymethyl group ($\text{R} = -\text{CH}_2-\text{S}-\text{CH}_2-\text{COOH}$), such as $\text{R} = -\text{CH}_2-\text{S}-\text{Hq}-\text{CH}_3$ (by methyl mercury acetate), $-\text{CH}_2-\text{S}-\text{CN}$ (by NTCB), or with $-\text{CH}_3$ (alanine, by site-directed mutagenesis), the smaller the R moiety, the larger the residual enzymatic activity of modified TBADH.

On the other hand, when R was $-\text{CH}_2-\text{OH}$ (serine, by site-directed mutagenesis), somewhat larger than in alanine but still smaller than $-\text{CH}_2-\text{SH}$ or $-\text{CH}_2-\text{S}-\text{CN}$, rather than decreasing, the enzymatic activity of TBADH increased by 150%. This phenomenon could be due to a more effective binding of NADP to Ser 203 than to the wild-type Cys 203, either by optimal spatial fitting or through a potential hydrogen bond of the serine hydroxyl group (rather than the cysteine sulfhydryl) or both.

The DTT-mediated liberation of the chemically modified TBADH from the bound radical (via thiol–disulfide exchange), thereby restoring enzymatic activity, indicated that the biradical modification of Cys 203 is reversible. The reversibility of this reaction is different from that of another oligomeric enzyme, acetylcholinesterase (AChE) from *Torpedo californica*. In this AChE, modifying a single cysteine SH with the stable nitroxyl biradical transforms the protein into a partially unfolded, molten globule state (Dolginova et al., 1992; Kreimer et al., 1994). Although DTT reverses the modification, the protein remains locked in an irreversible, molten globule state (Dolginova et al., 1992; Kreimer et al., 1994).

After the active site zinc of TBADH was removed by *o*-phenanthroline, both Cys 203 and a second cysteine, Cys 37, were quantitatively carboxymethylated rapidly. As the nitroxyl biradical also modified two cysteines in the apoenzyme, Cys 37 and Cys 203 are the most likely targets for the biradical modification as well. Although the modification rate was slower than that in the apoenzyme, the presence of the catalytic zinc in the holoenzyme, either in the presence or in the absence of NADP, did not prevent the chemical modification of its putative ligand Cys 37 (Bogin et al., 1997) by either iodoacetate or the biradical. This finding agrees with reports on other ADHs, in which the cysteine ligand(s) of the active site zinc were carboxymethylated in the native enzyme. Carboxymethylation of Cys 46 or Cys 174 of HLADH (Li & Vallee, 1963; Harris, 1964; Chadha & Plapp, 1984), Cys 43 of sorbitol dehydrogenase (Jeffery et al., 1981), and Cys 37 of TBADH (this study) abolished the catalytic activity of all three enzymes. In *S. sulfataricus*, on the other hand, alkylating Cys 38 enhanced the catalytic activity of ADH (Raia et al., 1996).

Because iodoacetate lacks absolute selectivity toward cysteine, both excess alkylating reagent and prolonged reaction times were avoided to minimize side reactions with other nucleophiles in the protein. But nevertheless, even during the short reaction time and using little excess reagent, conditions in which Cys 203 became quantitatively modified, Cys 37 became only partially (up to 30%) alkylated in native TBADH. By contrast, the stable biradical is highly selective toward cysteine. Prolonged incubation (>5 h) of TBADH with excess biradical resulted in the modification of two cysteines (by EPR measurements), and the loss of the active site zinc (<0.05 g atom/monomer).

Measuring the coupling between the radicals as a measure of the distance between them and learning about the conformation of a protein in solution is possible in a double spin-labeled protein, such as modified TBADH. This approach was recently used in rhodopsin, in which the main mechanism of coupling between the two radicals was a dipole–dipole interaction at low and room temperature (Yang et al., 1996). In a thermostable protein like TBADH, we anticipated that the increase in the rate of protein motion at high temperature would lead to a shift and narrowing of the EPR spectrum, yielding additional information about the interactions between the radicals. Indeed, at elevated temperatures (353 K), the EPR spectrum of TBADH, double labeled with nitroxyl radicals, showed signals that are characteristic of the biradicals and indications that a strong spin exchange had taken place (Luckhurst, 1976; Hanson et al., 1996). The intensity of the exchange depends on the overlap of unpaired nitroxide electron orbitals, and the frequency of the collisions increases with increasing temperatures. The EPR spectrum of the double-labeled TBADH at high temperature suggested that the two labels were in close proximity. For the biradicals, this coupling could be through-bonds or through-space. In TBADH, the two labeled cysteines, Cys 37 and

Cys 203, are separated by a large number of covalent bonds, thus eliminating through-bond contributions. The through-space interaction of the biradicals depends exponentially on the distance between the radical centers (Closs et al., 1992). Using the data obtained for double-labeled peptides (Hanson et al., 1996), we estimated the distances between the two labels in TBADH as ≤ 10 Å.

As TBADH is a tetramer, the spin exchange could have occurred between the radicals on either two labeled cysteines of the same subunit or two cysteines belonging to different subunits. That the interaction is intra-subunit is more likely for the following reason. In the HLADH–NAD complex, the nicotinamide ring is not bound to the active site zinc atom but rather puts one surface of the ring in contact with the zinc-coordinated cysteine sulfur atoms (Eklund, 1983; Eklund et al., 1984). In TBADH, the analogous ligand is Cys 37 (Bogin et al., 1997). Our biochemical and spectroscopy data revealed that NADP protected Cys 203 from chemical modification as well, and that the modification of each cysteine proceeded independently. Therefore, the distance between the two cysteines should not exceed the length of an extended NADP molecule (~ 20 Å) (Wilson et al., 1992). Although the conformation of NADP could be different in an NADP–ADH complex, this number could be taken as an upper value for the distance between Cys 203 and Cys 37. When also considering the size of two radical moieties, the value of ≤ 10 Å is small enough to support the proposition that an intra-subunit interaction occurs between the two biradicals.

Although folding of TBADH has not been studied, it could be proposed that the native conformation of the protein is in equilibrium with a different somewhat unfolded conformation(s). At room temperature, the equilibrium favors the native state (Haynie & Friere, 1993; Bay & Englander, 1996); however, at high temperature the equilibrium shifts and the population of partially unfolded states tends to increase. The spin exchange observed by us may characterize the mutual distribution of Cys 203 and Cys 37 in this non-native state and the intensity of the exchange signal reflects on the population of this state.

In native TBADH, Cys 283 and Cys 295 are in the reduced form. When the enzyme was heated at 75 °C for 30 min at neutral pH, however, these cysteines were oxidized to cystine without affecting the catalytic activity of TBADH. As the results of many studies have suggested that disulfide bonds confer rigidity and stability on certain proteins (Nakamura et al., 1978; Wetzel et al., 1988; Matsumura et al., 1989; Dohlman et al., 1990; Mely & Gerard, 1990; Kanaya et al., 1991), several investigators have attempted to introduce disulfides to enhance the thermal stability of proteins, using such methods as site-directed mutagenesis (Wetzel et al., 1988; Matsumura et al., 1989; Luckey et al., 1991) or grafting cysteine-containing peptides (Magonet et al., 1992), for example. To probe the function of the cystine 283–295 disulfide in TBADH, we used site-directed mutagenesis to replace Cys 283 with serine. The almost identical enzymatic activity and thermostability of the wild-type and the mutant enzymes indicated, however, that the disulfide bond of the oxidized form of TBADH is not essential for thermostability.

The method for the biradical modification of cysteines described here is very useful for structural studies of proteins and is advantageous over classical methods using other spin labels. In the classical approach, the protein is modified with a large excess (up to 100-fold) of the spin label, and the modified protein can be analyzed by EPR only after removing excess reagent (Morrisett, 1976). Our method, on the other hand, (a) allows direct assessment of the

modification rate; (b) provides direct information on the availability of SH groups; and (c) enables the assessment of the effects of substrates, inhibitors, cofactors, biological membranes, and others on the reactivity of sulfhydryls in proteins and in protein assembly.

Materials and methods

Materials

TBADH (specific activity of 84 U/mg) was isolated from *Thermoaerobacter brockii* as described previously (Peretz & Burstein, 1989). Apo-TBADH was prepared by dialyzing TBADH (1 mL of a solution of 5 mg/mL) against three changes of 100 mL of a solution containing 1,10-phenanthroline, 20 mM, pH 6.0, at 4 °C for a total of 24 h. Excess reagent was removed from the protein solution by gel filtration on a column of Bio-Gel P-2. The stable nitroxide biradical bis(1-oxy-2,2,5,5-tetramethyl-3-imidazoline-4-yl) disulfide, was synthesized as described previously (Khramtsov et al., 1989). Stock solutions of the reagent (0.5 M in acetonitrile) were stored at -20 °C. The mercury-containing stable radical, R^*Hg-Cl , was synthesized as described previously (Voldarsky & Weiner, 1983). Iodo[2- ^{14}C]acetic acid (2 mCi/mmol) was a product of Amersham (Buckinghamshire, UK). 2,2,6,6-Tetramethyl-1-piperidinyloxy sulfide was a product of Aldrich (Milwaukee, WI). TPCK-treated trypsin, β -nicotinamide adenine dinucleotide phosphate (NADP) monosodium salt, β -nicotinamide adenine dinucleotide phosphate reduced form (NADPH) tetrasodium salt, *d,l*-dithiothreitol (DTT), iodoacetic acid, 1,10-phenanthroline, and guanidine-HCl (Gdn-HCl) were purchased from Sigma (St. Louis, MO). 2-Nitro-5-thiocyanobenzoic acid (NTCB) and [^{14}C]NTCB were prepared according to Degani and Patchornik (Degani et al., 1970). Oligonucleotides were prepared with an Applied Biosystems model 380B DNA synthesizer. Restriction endonuclease, other enzymes required for cloning, [γ - ^{32}P]ATP, [α - ^{35}S]dATP, and [^{14}C]NaCN were obtained from Amersham Corp. (Buckinghamshire, UK).

Reduction and carboxymethylation

Reduction and S-carboxymethylation of TBADH was performed essentially as described by Crestfield (Crestfield et al., 1963), except that dithiothreitol (Cleland, 1964) was used in place of 2-mercaptoethanol. The protein (1 mg in 0.5 mL) was reacted for 30 min with a fourfold excess of DTT over each cysteine residue (four per subunit) in 0.5 M Tris-HCl, pH 8.0–8.5. Gdn-HCl (6 M) was used for complete protein denaturation. For S-carboxymethylation, either 2-iodoacetic acid or [2- ^{14}C]iodoacetic acid (2 mCi/mmol) was added (10–100 mM) for 30 min in the dark. The reaction was stopped by adding excess DTT, followed by exhaustive dialysis against 50 mM ammonium bicarbonate at 4 °C for a total of for 24 h.

Chemical modification of TBADH with nitroxide biradical and organomercurial radical

The protein in 50 mM Tris-HCl, pH 7.3, was treated with a solution of the nitroxide biradical in acetonitrile. The total volume of acetonitrile in the reaction mixture was $\leq 5\%$. To assess the degree of inactivation of TBADH, the reaction mixture containing TBADH (5 mg/mL) and the nitroxide biradical (0.5–10 mM) was first incubated at 37 °C and at specified time intervals, aliquots were

removed from the incubation mixture and diluted 100-fold into the enzyme spectrophotometric assay system.

To monitor the kinetics of the reaction, the reaction was initiated by mixing an aqueous solution of the nitroxide biradical (20 μ M, pH 7.3) with an equal volume of TBADH (4 μ M) in a mixer placed inside an EPR spectrometer resonator (mixing time ~ 15 s). The kinetics of the thiol–disulfide exchange between the nitroxide biradical and the sulfhydryl groups of TBADH was determined by following the appearance of the monoradical component in the EPR spectrum.

For chemical modification of reactive cysteines, TBADH (20 nmol in 1 mL) was reacted with the nitroxide biradical or organomercurial radical (4 mmol) at room temperature for five hours. Excess reagent was removed by gel filtration on a column of Bio-Gel P-2. Fractions corresponding to the chemically modified and inactive protein were combined and concentrated to a volume of 0.4 mL by ultra filtration, using Centricon-30 (Amicon Inc., Beverly, MA).

Enzyme activity assay

The enzymatic activity of TBADH was measured by monitoring the reduction of NADP $^{+}$ at 40 °C at 340 nm ($\epsilon_{340} = 6.2 \text{ mM}^{-1} \text{ cm}^{-1}$). For routine assays, the activity of the enzyme was determined in 100 mM Tris-HCl (pH 7.8) containing 150 mM 2-butanol and 0.5 mM NADP $^{+}$ in a total volume of 1 mL. One unit of TBADH was defined as the amount of enzyme that catalyzes the oxidation of 1 μ mol 2-butanol/min under the above standard conditions.

Proteolytic digestion of TBADH

The alkylated protein was first separated from excess reagents by exhaustive dialysis against 50 mM ammonium bicarbonate and then digested with trypsin, at a ratio (w/w) of S/E = 100, at 37 °C for five hours. Lysyl peptide bonds were selectively cleaved with *Achromobacter* protease I in 50 mM Tris-HCl/3 M urea, pH 9, at 37 °C for five hours, S/E = 300.

Site-directed mutagenesis of TBADH

The manipulation of DNA was carried out according to standard procedures (Sambrook et al., 1989). Cloning of the *T. brockii adh* gene, expression of recombinant TBADH (rTBADH) in *E. coli*, and purification of rTBADH and its mutants was performed as described previously (Peretz et al., 1997). The plasmid pBS-M105/2 used in these studies consisted of a pBluescript II SK(+) (Stratagene, San Diego, CA) vector and an insert composed of the DNA encoding the 352 amino acid residues of TBADH and two flanking regions of 249 nucleotides upstream from the initiation codon and 342 nucleotides downstream from the termination codon. Site-directed mutagenesis of rTBADH was performed according to the method of Kunkel (Kunkel et al., 1990).

Gel filtration and RPLC

Gel filtration on Bio-Gel P-2 (100–200 mesh, Bio-Rad Laboratories) was performed with an ISCO system. RPLC for protein and peptide fractionation was performed with Aquapore RP-300 columns (C-8, 4.6 $\times$ 200 mm) (Applied Biosystems, Foster City, CA) using a modular chromatography system of SpectraPhysics (Models SP- 8800/8780/8500/8773/4290, equipped with a Barspec Model

1937 Chrom-A-Scope fast scan detector). Proteins and the peptides were eluted from the columns by linear gradients of acetonitrile (5–40% in 30–45 min) in 0.1% aqueous trifluoroacetic acid, at a flow rate of 1 mL/min.

Analytical procedures

Protein concentration was determined according to Bradford (1976), using a commercial reagent (Bio-Rad Laboratories, Richmond, CA) and bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis was performed on 8–20% or 12% slab gels using a Bio-Rad MiniProtein II system. Staining was with Coomassie Brilliant Blue R. Amino acid analyses were performed with a Dionex D-400 amino acid analyzer (Dionex Co., Sunnyvale, CA), after hydrolysis in a Waters Pico-Tag station (Waters Associates, Milford, MA). The amino acid sequences of peptides were determined with an ABI Model 475A protein sequencer (PE-ABI division, Foster City, CA).

Electron Paramagnetic Resonance spectra were measured at room temperature on a Varian E-12 spectrometer or a Bruker 200 D-SRC spectrometer in a 200 μ L flat cell. For kinetic and temperature measurements 100 μ L quartz capillary was used. The concentration of the nitroxide radical covalently bound to TBADH was determined by double-integration of the EPR spectra, using 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO) for calibration.

Metal concentration was determined by atomic absorption spectroscopy with a Perkin-Elmer Model 5100BC spectrometer. Absorption spectroscopy was performed using a Beckman model DU 7000 spectrophotometer.

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