Fast purification of thioredoxin reductases and of thioredoxins with an unusual redox-active centre from anaerobic, amino-acid-utilizing bacteria

Claudia Harm$ Manfred A. Meyer$ and Jan R. Andreesen$2

Author for correspondence: Jan R. Andreesen. Tel: +49 345 55 2 63 50/51. Fax: +49 345 55 2 70 10. e-mail: j.andreesen@mikrobiologie.uni-halle.de

1 Institut für Mikrobiologie der Georg-August-Universität Göttingen, Grisebachstr. 8, 37077 Göttingen, Germany

2 Institut für Mikrobiologie der Martin-Luther-Universität Halle, Kurt-Mothes-Str. 3, 06099 Halle, Germany

Thioredoxin reductase and thioredoxin are primarily involved in catabolic metabolism as important electron carriers in anaerobic, amino-acid-degrading bacteria. A general and fast procedure was developed for the purification of thioredoxin reductase and thioredoxin from Eubacterium acidaminophilum, Clostridium litorale, C. sticklandii, C. sporogenes, C. cylindrosporum and 'Tissierella creatinophila' based upon their properties: the binding to 2',5'AMP-Sepharose by thioredoxin reductase and the inability of thioredoxins to bind to a DEAE-Sephacel column. The consensus sequence at the active site of thioredoxins (-WCGPC-) was found to be modified in all of these anaerobes: Trp-31 (Escherichia coli nomenclature) was replaced by Gly or Ser, Gly-33 by Val or Glu. None of these thioredoxins reacted with thioredoxin reductase of E. coli or vice versa, but they did interact with the thioredoxin reductases obtained from the other anaerobes studied. Based upon their distinguishing features it is suggested that these thioredoxins might form an evolutionarily separate group.

Keywords: thioredoxin, thioredoxin reductase, anaerobic, amino-acid-utilizing bacteria

INTRODUCTION

Many anaerobic, amino-acid-utilizing bacteria catalyse a Stickland-type reaction by which one amino acid is oxidized and usually proline or glycine (or its derivatives sarcosine and betaine) functions as electron acceptor via a specific reductase system (Seto, 1980; Andreesen, 1994a, b). These reductases are generally composed of at least three proteins, traditionally called P_A, P_B and P_C (Stadtman, 1978). Protein P_B contains selenocysteine (Kreimer & Andreesen, 1995); it is responsible for the substrate specificity and therefore differs in each reductase (Meyer et al., 1995). However, the reductases share the same P_A and P_C components (Dietrichs et al., 1991; Schröder & Andreesen, 1992). By the reductive deamination of glycine, sarcosine or betaine, the selenoprotein P_A becomes oxidized and has to be reduced by the thioredoxin system to start a new catalytic cycle (Dietrichs et al., 1991; Meyer et al., 1991; Andreesen, 1994a; Kreimer et al., 1997). In addition to its essential role as electron donor in these reductive deamination reactions of anaerobic organisms (Dietrichs et al., 1991; Meyer et al., 1995; Andreesen, 1994a, b), the thioredoxin system is generally involved in many biosynthetic or protein folding functions (Holmgren, 1985, 1989; Follmann & Hemberlein, 1995/96).

The thioredoxin system is composed of two proteins, an NADPH-dependent thioredoxin reductase and thioredoxin (Holmgren, 1989). Both are redox-active proteins with two cysteine residues, which are characteristically separated by two amino acids in the catalytic centre. Thioredoxin reductases are members of a family of pyridine nucleotide-disulfide oxidoreductases (Perham et al., 1988; Williams, 1992). In micro-organisms, thioredoxin reductase is generally a dimer of identical subunits with a molecular mass of 35 kDa, each subunit containing one redox-active disulfide and one tightly bound flavin adenine dinucleotide (FAD) (Mulrooney & Williams, 1994). The flavin mediates the transfer of reducing equivalents from NADPH to the disulfide bond. Thioredoxin is a ubiquitous protein with a...
molecular mass of 12 kDa (Buchanan et al., 1994) and two redox-active cysteine residues at an exposed active centre with the highly conserved amino acid sequence -WCGPC- (Eklund et al., 1991). In anaerobic, amino-acid-degrading bacteria using the Stickland reaction, the thioredoxin system interacts with selenoprotein -WCGPC- (Eklund et al., 1991). As previously observed for the thioredoxins of Eubacterium acidaminophilum and Clostridium litorale (Meyer et al., 1991), the Clostridium sticklandii, Clostridium sporogenes, Clostridium cylindrosporum and 'Tissierella creatinophila' thioredoxins stimulated the dihydrolipoamide dehydrogenase activity of the corresponding thioredoxin reductase. This property was used as the assay for the purification of thioredoxins and thioredoxin reductases (Meyer et al., 1991). We now report a fast, general procedure for the purification of these thioredoxin reductases and thioredoxins. In addition, we report that the thioredoxins of these anaerobic, amino-acid-utilizing bacteria substitute Trp-31 (Escherichia coli) of the highly conserved sequence -WCGPC- of the redox-active centre of the corresponding thioredoxin reductase. This property was used as the assay for the purification of thioredoxins and thioredoxin reductases (Meyer et al., 1991). We now report a fast, general procedure for the purification of these thioredoxin reductases and thioredoxins. In addition, we report that the thioredoxins of these anaerobic, amino-acid-utilizing bacteria substitute Trp-31 (Escherichia coli) of the highly conserved sequence -WCGPC- of the redox-active centre of the corresponding thioredoxin reductase. This property was used as the assay for the purification of thioredoxins and thioredoxin reductases (Meyer et al., 1991). We now report a fast, general procedure for the purification of these thioredoxin reductases and thioredoxins. 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applied to a Sephacryl S-100 HR column (2.6 x 100 cm; bed volume 496 ml) previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The proteins were eluted with the same buffer at a flow rate of 30 ml h⁻¹ and fractions with thioredoxin activity were pooled. The final yield was 48 mg thioredoxin in the case of ‘T. creatinophila’. For the purification of thioredoxin from C. sticklandii 120 ml crude extract (3840 mg protein) was diluted with Tris buffer (50 mM, pH 8.5, plus 1 mM EDTA) to yield a protein concentration of 10 mg ml⁻¹. The protein solution was incubated at 100 °C for 5 min and the purification continued with an acid treatment as described here.

To purify the thioredoxin reductase from the respective organisms, the protein pool with reductase activity after elution from DEAE-Sephacel was dialysed against 50 mM potassium phosphate buffer, pH 7-8, and then applied to a 2',5'-ADP-Sepharose column (bed volume 5/6 ml, flow rate 30 ml h⁻¹) previously equilibrated in this buffer. After being washed with buffer, the protein eluted with 500 mM KCl in equilibration buffer. Finally, the thioredoxin reductase was dialysed against 50 mM potassium buffer, pH 7-8, and stored at -20 °C. This procedure was practicable for the enzymes from E. acidaminophilum, C. sporogenes and C. cylindrosporum. In the case of ‘T. creatinophila’, thioredoxin reductase did not bind tightly to 2',5'-ADP-Sepharose under these conditions; better binding to ADP-Sepharose was observed by replacing the 50 mM potassium buffer, pH 7-8, with 10 mM potassium buffer, pH 7-0. Thioredoxin reductase from ‘T. creatinophila’ was eluted by a gradient from 0–1 mM NADP in 112 ml 10 mM potassium phosphate buffer, pH 7-0. Thioredoxin reductase eluted at 0-32 mM NADP in buffer. The final yield was 7 mg homogeneous thioredoxin reductase from ‘T. creatinophila’.

Protein analyses. Protein content was estimated by the method of Bradford (1976) using bovine serum albumin as standard. In addition the method of Elliot & Brewer (1978) was used. Protein spectrum analysis was carried out as described previously (Dietrichs & Andreason, 1990). For anaerobic titration of thioredoxin reductase, the method of Freudenberg et al. (1989) was used. Electrophoresis and molecular mass determination were performed according to the methods of Meyer et al. (1995). The isoelectric points were determined on Protein Phastgel (Serva) at 4 °C in the pH range 3-10 using 2.5 % ampholytes. The procedure was performed according to the manufacturer’s instructions.

RESULTS

Purification of thioredoxin reductases and thioredoxins

Thioredoxin reductases and thioredoxins were purified starting from the same batches of crude extract of the respective bacteria. Purification of thioredoxin from C. cylindrosporum, C. sporogenes, C. sticklandii and ‘T. creatinophila’ to apparent homogeneity (Fig. 1) was achieved using anion-exchange chromatography with DEAE-Sephacel, acid treatment (titration of the protein solution with 10 M HCl to pH 3-0), affinity chromatography with Procion Red and gel permeation chromatography. Representative purification protocols are given in Table 1. In contrast to most known thioredoxins and as is the case, for example, for the protein from Clostridium pasteurianum (Hammel & Buchanan, 1981), the proteins isolated in this study did not bind to DEAE-Sephacel (in some cases the protein from ‘T. creatinophila’ bound partly to this matrix). Consequently the design of a fast separation of thioredoxin reductases and thioredoxins was possible. All the thioredoxins examined were resistant towards an acid treatment, indicating that the isolated proteins were very stable. In the case of the thioredoxin from C. sticklandii, a heat treatment was performed in addition to the acid treatment. The thioredoxins from C. cylindrosporum, C. sporogenes, C. sticklandii and ‘T. creatinophila’ were bound tightly to the affinity gel material Procion Red and were eluted with 0.5 M KCl. Homogeneity was obtained by final separation on Sephacryl-100 HR. This general procedure, used for the purification of the thioredoxins from the above-mentioned bacteria, also worked for the purification of the proteins from the related species E. acidaminophilum and C. litorale, whose purification by other methods was described previously (Meyer et al., 1991). Several milligrams of homogeneous thioredoxins of the different organisms were obtained from each purification procedure.

Thioredoxin reductase was purified by applying the fractions with reductase activity following DEAE-Sephacel chromatography to an affinity column of 2',5'-ADP-Sepharose, which is often used for purification of NADP-dependent enzymes and has previously been used for thioredoxin reductases (Pigiet & Conley, 1977; Holmgren & Björnstedt, 1995). However, binding to this affinity matrix requires dithiols such as Cleland reagents to be absent. The thioredoxin reductases from C. sporogenes, C. cylindrosporum and ‘T. creatinophila’...
Table 1. Purification of thioredoxins from different anaerobic, amino-acid-utilizing bacteria

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Yield (mg protein) from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C. sticklandii</td>
</tr>
<tr>
<td>Crude extract</td>
<td>3940*</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>–</td>
</tr>
<tr>
<td>Acid treatment</td>
<td>144</td>
</tr>
<tr>
<td>Procion Red</td>
<td>9.5</td>
</tr>
<tr>
<td>S-100 HR</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*Crude extract of C. sticklandii was diluted with buffer before acid and heat treatment; no chromatography with DEAE-Sephacel was used.
†Thioredoxin bound partly to DEAE-Sephacel.

Table 2. Molecular masses and isoelectric points (pl) of thioredoxins and thioredoxin reductases from C. sticklandii, C. sporogenes, 'T. creatinophila' and C. cylindrosporum

<table>
<thead>
<tr>
<th>Organism</th>
<th>Molecular mass (kDa) of thioredoxin as determined by:</th>
<th>pl of thioredoxin</th>
<th>Molecular mass (kDa) of thioredoxin reductase as determined by:</th>
<th>pl of thioredoxin reductase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SDS-PAGE</td>
<td>Sephacryl-100 HR</td>
<td>Sephadex G 75</td>
<td>SDS-PAGE</td>
</tr>
<tr>
<td>C. sticklandii</td>
<td>13-0</td>
<td>20-0</td>
<td>17-0</td>
<td>4-5</td>
</tr>
<tr>
<td>C. sporogenes</td>
<td>13-5</td>
<td>17-0</td>
<td>15-5</td>
<td>6-75</td>
</tr>
<tr>
<td>'T. creatinophila'</td>
<td>13-6</td>
<td>18-0</td>
<td>ND</td>
<td>4-65</td>
</tr>
<tr>
<td>C. cylindrosporum</td>
<td>13-0</td>
<td>16-0</td>
<td>ND</td>
<td>5-0</td>
</tr>
</tbody>
</table>

ND, Not determined.

Molecular mass and isoelectric points

A molecular mass of approximately 13.0 kDa was estimated, by SDS gradient PAGE, for the thioredoxins from C. sticklandii, C. sporogenes, C. cylindrosporum and 'T. creatinophila' (Table 2). The native proteins were found to have molecular masses of about 17 kDa for C. sticklandii, 15.5 kDa for C. sporogenes, 18 kDa for 'T. creatinophila' and 16 kDa for C. cylindrosporum following gel filtration chromatography on Sephadex G 75 and Sephacryl-100 HR. Consequently, the thioredoxins of these anaerobes were monomers while the thioredoxin of E. coli could be observed as a monomer (13 kDa) and a dimer (23-5 kDa) (Fig. 1).

The apparent molecular mass of the subunit from thioredoxin reductases was, according to SDS-PAGE, about 31 kDa (C. sporogenes), 33 kDa (C. cylindrosporum), 36 kDa (C. sticklandii) and 33 kDa ('T. creatinophila'), respectively (Table 2). Data obtained by native PAGE indicated that all the thioredoxin reductases examined were homodimers with apparent molecular masses of 58 kDa (C. sporogenes), 70 kDa (C. sticklandii), 58 kDa (C. cylindrosporum) and 66 kDa ('T. creatinophila') (data not shown).
Table 3. Comparison of N-terminal amino acid sequence data of the thioredoxins from *Eubacterium acidaminophilum*, *C. litorale*, *C. sticklandii*, *C. sporogenes*, *C. cylindrosporum*, *T. creatinophila* and *Escherichia coli*

Consensus amino acids are underlined. The N-terminal sequences of the thioredoxins from *C. sporogenes*, *C. sticklandii* and *T. creatinophila* have been deposited in the SWISS-PROT protein sequence database under the accession numbers P81108, P81109 and P81110, respectively.

<table>
<thead>
<tr>
<th>Consensus*</th>
<th>1 ...... 10</th>
<th>D</th>
<th>F</th>
<th>T</th>
<th>VL</th>
<th>20 ......</th>
<th>G</th>
<th>LVDFWA</th>
<th>WCGPC</th>
<th>K</th>
<th>MIA</th>
<th>P</th>
<th>30 ......</th>
<th>ILDEIADE</th>
<th>40 ......</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>SDKIHLTDD</td>
<td>SFDPDVKLAD</td>
<td>GAILVDFWAE</td>
<td>WCGPC</td>
<td>KMIAP</td>
<td>ILDEIADE</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td><em>E. acidaminophilum</em></td>
<td>MSALLVEIDKQ</td>
<td>QGQAVLVEAE</td>
<td>GYVLVDYFSD</td>
<td>GCVFKALMP</td>
<td>DVEEL</td>
<td></td>
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<td></td>
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<td></td>
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<tr>
<td><em>C. litorale</em></td>
<td>MMLDKE</td>
<td>TLRPVLQGT</td>
<td>GYVLDFDSE</td>
<td>GCVFKALMP</td>
<td>AVEELSKK</td>
<td></td>
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<tr>
<td><em>C. sticklandii</em></td>
<td>MFLDKD</td>
<td>TFETPVLOGT</td>
<td>GYVLDFDSE</td>
<td>GSEFSPKALMP</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td><em>C. sporogenes</em></td>
<td>MVLDDK</td>
<td>TFEEVQLTK</td>
<td>GYVLDFYGD</td>
<td>GSVFSEALMP</td>
<td>DVE</td>
<td></td>
<td></td>
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<tr>
<td><em>T. creatinophila</em></td>
<td>MIELDKS</td>
<td>NFEVVLKAE</td>
<td>GTVLDFESP</td>
<td>SSEPSSKALMP</td>
<td>HVHDFEE</td>
<td></td>
<td></td>
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† Holmgren (1968); Lim *et al.* (1987).
§ Kreimer *et al.* (1997).

Table 4. Reaction of thioredoxins from anaerobic, amino-acid-utilizing bacteria with different thioredoxin reductases

<table>
<thead>
<tr>
<th>Source of thioredoxin</th>
<th>Activity [U (mg thioredoxin)^{-1}] of thioredoxin reductase* from:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td><em>E. coli</em></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>37</td>
</tr>
<tr>
<td><em>C. litorale</em></td>
<td>0</td>
</tr>
<tr>
<td><em>E. acidaminophilum</em></td>
<td>0</td>
</tr>
<tr>
<td><em>C. sporogenes</em></td>
<td>0</td>
</tr>
<tr>
<td><em>T. creatinophila</em></td>
<td>0</td>
</tr>
<tr>
<td><em>C. cylindrosporum</em></td>
<td>0</td>
</tr>
<tr>
<td><em>C. sticklandii</em></td>
<td>0</td>
</tr>
</tbody>
</table>

*Figures in bold represent the reactions of the homologous proteins.*

The isoelectric points (pI) were calculated for the thioredoxins and several thioredoxin reductases from these anaerobes (Table 2). All proteins examined were acidic, with pI values in the range 4.5–6.75.

**N-terminal sequence analysis of thioredoxins**

In Table 3 the N-terminal amino acid sequences determined for the thioredoxins from *C. sticklandii*, *C. sporogenes* and *T. creatinophila* are compared to the sequences derived from the sequences of the corresponding genes of *Escherichia coli*, *Eubacterium acidaminophilum* and *C. litorale*. No data were obtained for the thioredoxin from *C. cylindrosporum*. Although the N-terminal sequence analysis of the isolated thioredoxins exhibited a high degree of identity to the derived consensus amino acids (Eklund *et al.*, 1991), important changes were noted, especially close to the redox-active centre. The highly conserved Trp-31 and Gly-33 (amino acid positions refer to the *E. coli* thioredoxin) were not present in these thioredoxins: the aromatic Trp-31 was replaced by the small amino acid Gly (*C. sticklandii*, *C. sporogenes*) or Ser (*T. creatinophila*). In addition, the Gly-33 was replaced by Val (*C. sporogenes*, *E. acidaminophilum*, *C. litorale*) or Glu (*C. sticklandii* and *T. creatinophila*). The latter exchange has not been reported previously, but it is also found in the sequence of the corresponding gene from *C. sticklandii* (A. Gräntzdörffer, personal communication).

**Enzyme activities of thioredoxin reductase and thioredoxin**

Thioredoxin reductase and thioredoxin were measured using a complementation assay in which NADPH formation from DTT was determined. To ensure that
the activities did not result from a dihydrolipoamide dehydrogenase, which is also present in most of these organisms (Dietrichs et al., 1990), the active fractions were tested with dihydrolipoamide and NADP. The thioredoxins or thioredoxin reductases isolated from these anaerobic bacteria did not react with the thioredoxin reductase or thioredoxin of E. coli, respectively. However, the respective proteins isolated from the other anaerobes reacted with each other, causing a maximal change in activity of about two- or threefold (Table 4). We conclude that the proteins were interchangeable within this distinct group.

We also determined the ability of different thioredoxin reductases to catalyse the reduction of insulin by DTT (Fig. 3), as thioredoxin can act as a general protein disulfide reductase (Gleason & Holmgren, 1988). The reduction of insulin leads to a reduction of the two interchain disulfide bonds and consequently causes precipitation of the protein. The reaction with insulin was catalysed by all thioredoxins tested (Fig. 3). Thioredoxin from E. coli catalysed a fast reduction of insulin whereas thioredoxins from the anaerobic organisms were slower (30–40 min) to start with this reaction. Thus, the observed deviations in the redox-reactive centre might cause a modification in biochemical reactivity.

The absorption spectra of the thioredoxin reductase from C. cylindrosporum, C. sporogenes, C. sticklandii and ‘T. creatinophila’ were quite similar to those reported for E. acidaminophilum and C. litorale (Dietrichs et al., 1990, 1991). As an example, the absorption spectra of the thioredoxin reductase of ‘T. creatinophila’ that was reduced by NADPH are shown in Fig. 4. In all cases, the reduction of the FAD by NADPH required an excess of reducing equivalents and led to a semiquinone form as indicated by a broad peak at 585 nm.

**DISCUSSION**

In anaerobic, amino-acid-degrading bacteria catalysing Stickland reactions, biochemical and molecular genetic studies have indicated that the thioredoxin system is the primary electron donor for glycine and betaine reduction (Stadtman, 1978; Stadtman & Davis, 1991; Lübbers & Andreesen, 1993; Andreesen, 1994b; Kreimer & Andreesen, 1995; Meyer et al., 1995; Kreimer et al., 1997). The substrate-specific selenoproteins P_B seem to play the central role in substrate attack and conversion by interacting with the respective substrate to form a P_B-bound, Se-carboxymethylselenoether that is later transferred to the small 16 kDa selenoprotein P_A. The selenoprotein P_B contains the motif -UXXCXXC-(Kreimer & Andreesen, 1995), reminiscent of selenoprotein P_A and thioredoxin. The P_A-bound carboxymethylselenoether has been shown to react with protein P_C to give acetylphosphate and oxidized protein P_A (Arkowitz & Abeles, 1991; Stadtman & Davis, 1991; Schräder & Andreesen, 1992). The latter is finally reduced by thioredoxin and thioredoxin reductase. Antibodies raised against thioredoxin reductase of *Eubacterium acidaminophilum* cross-react with the thioredoxin reductases of C. litorale, C. cylindrosporum, C. sticklandii, C. sporogenes and ‘T. creatinophila’. This latter enzyme was also formerly called electron-transferring protein, due to its inactivity with thioredoxin of *Escherichia coli* (Dietrichs et al., 1990; Harms, 1995). The similarities of these proteins have
been partially documented from the N-terminal sequences of *E. acidaminophilum, C. litorale, C. sporogenes* and *C. sticklandii* (Dietrichs et al., 1990; Kreimer & Andreesen, 1995). In contrast to the observed promiscuity of thioredoxins from other groups of organisms (Holmgren & Björnstedt, 1995), the components of the thioredoxin system from the anaerobic, amino-acid-degrading bacteria formed an enzymically active complex only if interchanged within their group, and not with the respective proteins obtained from *E. coli*. In addition, thioredoxin from *E. coli* is completely inactive as an electron donor for glycine reduction (Dietrichs et al., 1991) and antibodies raised against thioredoxin of *E. acidaminophilum* cross-react with corresponding proteins of other anaerobic bacteria, but not with the thioredoxin from *E. coli* (Andreesen, 1994a). Thus, thioredoxin reductases and thioredoxins from the anaerobic, amino-acid-degrading bacteria analysed are highly similar at the enzymological and immunological levels, although the organisms from which they derive belong to different phylogenetic clusters of the clostridia (Collins et al., 1994).

The thioredoxins obtained from these anaerobes deviate characteristically at the redox-active centre from the published consensus sequence. The -WCGPC- motif of thioredoxins (Holmgren, 1968, 1985, 1989; Eklund et al., 1991) is substituted by -GCVPC- in *Eubacterium acidaminophilum, C. litorale* (Meyer et al., 1991; Lübbers & Andreesen, 1993; Kreimer et al., 1997) and *C. sporogenes*. ‘*T. creatinophila*’ exhibited two so far unknown substitutions: Trp-31 was replaced by Ser instead of Gly, as found for the thioredoxins of the other anaerobes of this group. In the case of ‘*T. creatinophila*’ and *C. sticklandii* Gly-33 was substituted by Glu instead of Val, as found for the other members of this group. The importance of the unusual Gly-31 for catalysis has been shown by site-directed mutagenesis using *C. litorale* thioredoxin (Kreimer et al., 1997): if it is replaced by tryptophan, the mutated thioredoxin shows only 5% of its original activity, but still fails to interact with the thioredoxin reductase of *Escherichia coli*. Thus, a substitution of tryptophan by a less bulky amino acid such as glycine or serine might be of advantage, for example by allowing a better interchange with the mixed sulfide-selenide of protein *P*<sub>s</sub> of anaerobic bacteria containing glycine reductase. The proteins of the thioredoxin system obtained from ‘*T. creatinophila*’ were enzymically less effective than those from other anaerobic bacteria if used in a heterologous system, perhaps reflecting unconventional exchanges. Considering all amino acid substitutions observed in otherwise conserved residues, it seems to be reasonable that no immunological or physiological relationship could be detected to the thioredoxin of *E. coli*. Taking all these differences into consideration, the data reported for thioredoxins from these anaerobic bacteria are further indications that they seem to represent a distinct line in evolution as indicated by the dendrogram representing only *E. acidaminophilum* and *C. litorale* (Kreimer et al., 1997).

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**REFERENCES**


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