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

Claudia Harms,¹ Manfred A. Meyer¹ and Jan R. Andreessen²

Author for correspondence: Jan R. Andreessen. Tel: +49 345 55 2 63 50/51. Fax: +49 345 55 2 70 10.
e-mail: j.andreessen@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-Sepharose 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; Andreessen, 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 & Andreessen, 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 & Andreessen, 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; Andreessen,

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; Andreessen, 1994a, b), the thioredoxin system is generally involved in many biosynthetic or protein folding functions (Holmgren, 1985, 1989; Follmann & Häberlein, 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

Abbreviation: FAD; flavin adenine dinucleotide.

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 P_A, containing a -CFVU- motif at the redox-active centre (Dietrichs *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 cylindrosporium* 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* nomenclature) of the highly conserved sequence -WCGPC- of the redox-active centre (Eklund *et al.*, 1991) with Gly or Ser, whereas Gly-33 was replaced by Val or Glu. The motif -SCEPC-, as detected in the thioredoxin of '*T. creatinophila*', has not been reported before, and supports the idea that these thioredoxins form an evolutionarily separate group.

METHODS

Chemicals. Enzymes and coenzymes were purchased from Boehringer Mannheim. Molecular mass marker kits were obtained from Sigma. DEAE-Sephacel, Sephacryl-100 HR, and 2',5'-ADP-Sepharose were from Pharmacia; Procion Red was purchased from ICI. Thioredoxin reductase of *E. coli* was kindly provided by C. H. Williams, Jr (Department of Biological Chemistry, University of Michigan, Ann Arbor, MI, USA) and thioredoxin from *E. coli* was obtained from Serva. All other chemicals were of the highest purity available from commercial sources.

Organisms and growth conditions. '*Tissierella creatinophila*' (DSM 6911) (a new species distinct from *T. creatinini*, not yet formally described; authors' unpublished work) was cultivated anaerobically on a medium containing 50 mM creatine and 40 mM sodium formate as substrates (Schleicher, 1990). Conditions of growth of the other species were as described previously: *Clostridium sticklandii* (DSM 519, ATCC 12662) was grown using 20 mM arginine, 20 mM serine, 20 mM threonine and 20 mM lysine as substrates (Wagner & Andreesen, 1995); *C. sporogenes* (DSM 633) was grown on 50 mM betaine and 25 mM glycine (Naumann *et al.*, 1983); and *C. cylindrosporium* HC-1 (DSM 605, ATCC 7906) was cultured with uric acid (12 mM) and glycine (40 mM) as substrates (Dürre & Andreesen, 1983). *Eubacterium acidaminophilum* (DSM 3953) was grown anaerobically on a medium containing 50 mM glycine (Zindel *et al.*, 1988).

Cell-extract preparation. The cells were mass-cultured in 20-litre carboys at 30 °C and harvested by centrifugation (8000 g) while they were in the exponential growth phase, frozen and stored at -20 °C. Cells (1 g) were resuspended in 1 ml 50 mM potassium phosphate, pH 7.8, then lysozyme (1 mg ml⁻¹), DNase I (0.1 mg per g cells), and PMSF (0.2 M in 2-propanol; 1 µl ml⁻¹) were added. The cell suspension was incubated at

37 °C for 30 min. The suspensions of *C. sticklandii*, *C. sporogenes*, *C. cylindrosporium* and *E. acidaminophilum* were passed twice through a precooled French pressure cell at 147 MPa (Dietrichs *et al.*, 1990) whereas the cells of '*T. creatinophila*' were frozen again at -20 °C after three initial passages through the French pressure cell. Lysozyme, DNase I and PMSF were added and incubated a second time; after which the cells (4 °C) were again passed six to ten times through the French pressure cell at 147 MPa. The cell debris was removed by centrifugation for 30 min at 10000 g at 4 °C. After ultracentrifugation (90 min, 100000 g) the resulting supernatant (crude extract) was used for enzyme preparation.

Enzyme assays. The activity of thioredoxin or thioredoxin reductase was measured spectrophotometrically in a complementation assay as described previously (Meyer *et al.*, 1991). To ensure that the enzyme activity did not result from the presence of a dihydrolipoamide dehydrogenase, the fractions were also tested with dihydrolipoamide and NADP (Dietrichs *et al.*, 1990). The activity of thioredoxins was also tested by the reduction of insulin (Holmgren, 1979). One unit is defined as the amount of enzyme which converts 1 µmol substrate min⁻¹ at 37 °C.

General procedure for purification of thioredoxin and thioredoxin reductase. All purification steps were performed at 4 °C. Both thioredoxin and thioredoxin reductase were purified starting from the same crude extract. The purification of thioredoxin and thioredoxin reductase of '*T. creatinophila*' is described below as a representative example. The purification steps for the proteins from *C. sticklandii*, *C. sporogenes* and *C. cylindrosporium* were nearly identical. No dithioerythritol or EDTA was added because a purification of thioredoxin reductase by chromatography on 2',5'-ADP-Sepharose was only possible without such additives. For purification, 117 ml crude extract of '*T. creatinophila*' (2493 mg protein) was applied with a flow rate of 60 ml h⁻¹ to a column of DEAE-Sephacel (5 × 30 cm; bed volume 132 ml), previously equilibrated with 50 mM potassium phosphate buffer, pH 7.8. Proteins that did not bind were eluted by washing the column with phosphate buffer. Thioredoxins from anaerobic, amino-acid-utilizing bacteria examined so far did not bind tightly to DEAE-Sephacel (Meyer *et al.*, 1991), with the possible exception of the thioredoxin of '*T. creatinophila*', whereas the corresponding thioredoxin reductases bound tightly to this gel material. In the case of extracts from '*T. creatinophila*', the column was washed with 630 ml buffer (flow rate 60 ml h⁻¹) until no protein was detectable. Bound proteins were eluted by a gradient of 0–1 M NaCl in 1400 ml 50 mM potassium phosphate buffer, pH 7.8, at a flow rate of 60 ml h⁻¹. Fractions (9.4 ml) containing thioredoxin or thioredoxin reductase activity were pooled. For further purification of thioredoxin, 249 ml protein solution (408 mg protein) was stirred on ice and titrated by the addition of concentrated HCl to pH 3.0. After an incubation of 10 min, the solution was neutralized with 10 M KOH. The precipitate was removed by centrifugation for 30 min at 30000 g. The supernatant was dialysed against 10 mM potassium phosphate buffer, pH 7.0 (336 mg protein in 260 ml), and applied to a Procion Red column (5 × 30 cm; bed volume 150 ml), previously equilibrated with 10 mM potassium phosphate buffer, pH 7.0, with a flow rate of 18 ml h⁻¹. After being washed with 150 ml of the same buffer, thioredoxin activity eluted with 0.5 M NaCl in 50 mM potassium phosphate buffer, pH 7.0. Fractions with enzyme activity were pooled (97 ml with 0.085 mg protein ml⁻¹), dialysed against 10 mM potassium phosphate buffer, pH 7.0, concentrated by lyophilization and dissolved in 2 ml H₂O. Finally the protein solution was

applied to a Sephacryl S-100 HR column (2.6 × 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 4.8 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 & Andreesen, 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 Precotes (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,

Fig. 1. SDS gradient PAGE (10–27.5%) and silver-stain of purified thioredoxins from different anaerobic, amino-acid-utilizing bacteria; 1 µg of each protein was applied. Lanes: 1, marker proteins; 2, *C. sporogenes*; 3, *C. litorale*; 4, *C. cylindrosporum*; 5, *Eubacterium acidaminophilum*; 6, *Escherichia coli*; 7, '*T. creatinophila*'.

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. creatino-*

Table 1. Purification of thioredoxins from different anaerobic, amino-acid-utilizing bacteria

Purification step	Yield (mg protein) from:				
	<i>C. sticklandii</i>	<i>C. sporogenes</i>	<i>C. cylindrosporum</i>	<i>E. acidaminophilum</i>	' <i>T. creatinophila</i> '
Crude extract	3940*	1175	1800	21000	2493
DEAE-Sephacel	—	747	270	4162	408†
Acid treatment	144	83	97	533	336
Procion Red	9.5	7.2	13	14.4	8
S-100 HR	1.4	6.7	8	8.3	4.8

* 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.

Fig. 2. SDS gradient PAGE (10–27.5%) and silver-stain of purified thioredoxin reductases from different anaerobic, amino-acid-utilizing bacteria; 0.5 µg of each protein was applied. Lanes: 1, '*T. creatinophila*'; 2, *Escherichia coli*; 3, *Eubacterium acidaminophilum*; 4, *C. cylindrosporum*; 5, *C. litorale*; 6, *C. sporogenes*; 7, marker proteins.

phila' were purified to apparent homogeneity by a two-step purification procedure revealing a single protein band in SDS-PAGE (Fig. 2). All thioredoxins and

thioredoxin reductases retained about 90% of their activities when stored at -20°C for three months.

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

Organism	Molecular mass (kDa) of thioredoxin as determined by:			pI of thioredoxin	Molecular mass (kDa) of thioredoxin reductase as determined by:		pI of thioredoxin reductase
	SDS-PAGE	Sephacryl-100 HR	Sephadex G 75		SDS-PAGE	Native PAGE	
<i>C. sticklandii</i>	13.0	20.0	17.0	4.5	36	70	ND
<i>C. sporogenes</i>	13.5	17.0	15.5	6.75	31	58	5.0
' <i>T. creatinophila</i> '	13.6	18.0	ND	4.65	33	66	4.75
<i>C. cylindrosporum</i>	13.0	16.0	ND	5.0	33	58	ND

ND, Not determined.

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.

Consensus*	1.....10	20	30	40	
	<u>D</u>	<u>F T VL</u>	<u>G LVDFWA</u>	<u>WCGPCK P</u>	
<i>E. coli</i> †	SDKIIHLTDD	SFDTDVLKAD	GAILVDFWAE	WCGPCKMIAP	ILDEIADE
<i>E. acidaminophilum</i> ‡	MSALLVEIDKD	QFQAEVLEAE	GYVLVDYFSD	GCVPCCKALMP	DVEEL
<i>C. litorale</i> §	MLMLDKD	TFKTEVLEGT	GYVLVDYYS	GCVPCCKALMP	AVEELSKK
<i>C. sticklandii</i>	MFELDKD	TFETEVLQGT	GYVLVDFWSE	G\$EP\$KALMP	
<i>C. sporogenes</i>	MLVLDDK	T\$EEEV\$KTK	GYVLVDYFGD	G\$VP\$EALMP	DVE
' <i>T. creatinophila</i> '	MIELDKS	N\$EEEV\$LKAE	GTVLVDFWSP	S\$EP\$KALMP	HVHDFEE

* Consensus: highly conserved residues in thioredoxins of different species according to Gleason & Holmgren (1988) and Eklund *et al.* (1991).

† Holmgren (1968); Lim *et al.* (1987).

‡ Lübbers & Andreessen (1993).

§ Kreimer *et al.* (1997).

\$ No signal obtained, but probably cysteine due to the method used.

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

Source of thioredoxin	Activity [U (mg thioredoxin) ⁻¹] of thioredoxin reductase* from:					
	<i>E. coli</i>	<i>C. litorale</i>	<i>E. acidaminophilum</i>	<i>C. sporogenes</i>	' <i>T. creatinophila</i> '	<i>C. cylindrosporum</i>
<i>E. coli</i>	3.7	0	0	0	0	0
<i>C. litorale</i>	0	6.9	7.4	6.3	1.9	4.8
<i>E. acidaminophilum</i>	0	4.2	3.7	4.5	0.4	3.4
<i>C. sporogenes</i>	0	3.7	4.0	6.9	0.6	4.2
' <i>T. creatinophila</i> '	0	1.1	3.0	3.4	3.0	1.4
<i>C. cylindrosporum</i>	0	5.0	7.7	5.8	0.7	3.7
<i>C. sticklandii</i>	0	2.1	3.2	4.5	0.5	2.1

* 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

Fig. 3. Reduction of insulin by thioredoxins from different organisms. ■, *Escherichia coli*; △, *Eubacterium acidaminophilum*; ●, *C. sporogenes*; ▲, *C. cylindrosporium*; □, '*T. creatinophila*'; ○, DTT.

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-active centre might cause a modification in biochemical reactivity.

The absorption spectra of the thioredoxin reductase from *C. cylindrosporium*, *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

Fig. 4. Absorption spectra of the thioredoxin reductase from '*T. creatinophila*'. Spectra were taken under a nitrogen atmosphere. The enzyme solution (1 mg ml⁻¹) was dialysed against anaerobic 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA. The oxidized protein (—) was reduced by one (---), three (·····) and five (-·-·-) equivalents NADPH in 50 mM potassium phosphate buffer, pH 7.8.

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; Schröder & 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. cylindrosporium*, *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_A 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

- Andreesen, J. R. (1994a). Acetate via glycine: a different form of acetogenesis. In *Acetogenesis*, pp. 568–629. Edited by H. L. Drake. New York/London: Chapman & Hall.
- Andreesen, J. R. (1994b). Glycine metabolism in anaerobes. *Antonie Leeuwenhoek* 66, 223–237.
- Arkowitz, R. A. & Abeles, R. H. (1991). Mechanism of action of clostridial glycine reductase: isolation and characterization of a covalent acetyl enzyme intermediate. *Biochemistry* 30, 4090–4097.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72, 248–254.
- Buchanan, B. B., Schurmann, P., Decottignies, P. & Lozano, R. M. (1994). Perspectives in biochemistry and biophysics. Thioredoxin: a multifunctional regulatory protein with a bright future in technology and medicine. *Arch Biochem Biophys* 314, 257–260.
- Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H. & Farrow, J. A. (1994). The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int J Syst Bacteriol* 44, 812–826.
- Dietrichs, D. & Andreesen, J. R. (1990). Purification and comparative studies of dihydrolipoamide dehydrogenases from anaerobic, glycine-utilizing bacteria *Peptostreptococcus glycinophilus*, *Clostridium cylindrosporum*, and *Clostridium sporogenes*. *J Bacteriol* 172, 243–251.
- Dietrichs, D., Meyer, M., Schmidt, B. & Andreesen, J. R. (1990). Purification of NADPH-dependent electron-transferring flavoproteins and N-terminal protein sequence data of dihydrolipoamide dehydrogenases from anaerobic, glycine-utilizing bacteria. *J Bacteriol* 172, 2088–2095.
- Dietrichs, D., Meyer, M., Rieth, M. & Andreesen, J. R. (1991). Interaction of selenoprotein P_A and the thioredoxin system, components of the NADPH-dependent reduction of glycine in *Eubacterium acidaminophilum* and *Clostridium litorale*. *J Bacteriol* 173, 5983–5991.
- Dürre, P. & Andreesen, J. R. (1983). Purine and glycine metabolism by purinolytic clostridia. *J Bacteriol* 154, 192–195.
- Eklund, H., Gleason, F. K. & Holmgren, A. (1991). Structural and functional relations among thioredoxins of different species. *Protein Struct Funct Genet* 11, 13–28.
- Elliot, J. & Brewer, J. M. (1978). The inactivation of the yeast enolase by 2,3-butanedione. *Arch Biochem Biophys* 190, 351–357.
- Follmann, H. & Haeberlein, I. (1995/96). Thioredoxins: universal, yet specific thiol–disulfide redox cofactors. *BioFactors* 5, 147–156.
- Freudenberg, W., Dietrichs, D., Lebertz, H. & Andreesen, J. R. (1989). Isolation of an atypically small lipoamide dehydrogenase involved in the glycine decarboxylase complex from *Eubacterium acidaminophilum*. *J Bacteriol* 171, 1346–1354.
- Gleason, F. K. & Holmgren, A. (1988). Thioredoxin and related proteins in prokaryotes. *FEMS Microbiol Lett* 54, 271–298.
- Hammel, K. E. & Buchanan, B. B. (1981). Thioredoxin and ferredoxin-thioredoxin reductase activity occur in a fermentative bacterium. *FEBS Lett* 130, 88–92.
- Harms, C. (1995). *Anaerobier Kreatin-Stoffwechsel in Tissierella creatinophila: Anreicherung und Charakterisierung beteiligter*

Proteine, besonders von verschiedenen Komponenten der Sarkosin-Reduktase. PhD thesis, Universität Göttingen.

Holmgren, A. (1968). Thioredoxin. 6. The amino acid sequence of the protein from *Escherichia coli* B. *Eur J Biochem* **6**, 475–484.

Holmgren, A. (1979). Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. *J Biol Chem* **254**, 9627–9632.

Holmgren, A. (1985). Thioredoxin. *Annu Rev Biochem* **54**, 237–271.

Holmgren, A. (1989). Thioredoxin and glutaredoxin systems. *J Biol Chem* **264**, 13963–13966.

Holmgren, A. & Björnstedt, M. (1995). Thioredoxin and thiorredoxin reductase. *Methods Enzymol* **252**, 199–208.

Kreimer, S. & Andreesen, J. R. (1995). Glycine reductase of *Clostridium litorale*. Cloning, sequencing, and molecular analysis of the *grdAB* operon that contains two in-frame TGA codons for selenium incorporation. *Eur J Biochem* **234**, 192–199.

Kreimer, S., Söhling, B. & Andreesen, J. R. (1997). Two closely linked genes encoding thioredoxin and thioredoxin reductase in *Clostridium litorale*. *Arch Microbiol* **168**, 328–337.

Lim, C. J., Fuchs, J. A., McFarlan, S. C. & Hogenkamp, H. P. (1987). Cloning, expression, and nucleotide sequence of a gene encoding a second thioredoxin from *Corynebacterium nephridii*. *J Biol Chem* **262**, 12114–12119.

Lübbers, M. & Andreesen, J. R. (1993). Components of glycine reductase from *Eubacterium acidaminophilum*: Cloning, sequencing and identification of the genes for thioredoxin reductase, thioredoxin and selenoprotein P_A. *Eur J Biochem* **217**, 791–798.

Meyer, M., Dietrichs, D., Schmidt, B. & Andreesen, J. R. (1991). Thioredoxin elicits a new dihydrolipoamide dehydrogenase with the electron-transferring flavoprotein in *Clostridium litorale* and *Eubacterium acidaminophilum*. *J Bacteriol* **173**, 1509–1513.

Meyer, M., Granderath, K. & Andreesen, J. R. (1995). Purification and characterization of protein P_B of betaine reductase and its relationship to the corresponding proteins glycine reductase and sarcosine reductase from *Eubacterium acidaminophilum*. *Eur J Biochem* **234**, 184–191.

Mulrooney, S. B. & Williams, C. H., Jr (1994). Potential active-site base of thioredoxin reductase from *Escherichia coli*: examination of histidine-245 and aspartate-139 by site-directed mutagenesis. *Biochemistry* **33**, 3148–3154.

Naumann, E., Hippe, H. & Gottschalk, G. (1983). Betaine: new oxidant in the Stickland reaction and methanogenesis from betaine and L-alanine by a *Clostridium sporogenes*–*Methanosarcina barkeri* coculture. *Appl Environ Microbiol* **45**, 474–483.

Perham, R. N., Berry, A. & Scrutton, N. S. (1988). Flavoprotein disulfide oxidoreductases: protein engineering of glutathione reductase from *Escherichia coli*. *Biochem Soc Trans* **16**, 84–87.

Pigiet, V. P. & Conley, R. R. (1977). Purification of thioredoxin, thioredoxin reductase, and glutathione reductase by affinity chromatography. *J Biol Chem* **252**, 6367–6372.

Schleicher, A. (1990). *Anaerobier Abbau von Kreatinin und Kreatin unter Beteiligung von Reduktase-Reaktionen*. Diploma thesis, Universität Göttingen.

Schröder, T. & Andreesen, J. R. (1992). Purification and characterization of protein P_C, a component of glycine reductase from *Eubacterium acidaminophilum*. *Eur J Biochem* **206**, 79–85.

Seto, B. (1980). The Stickland reaction. In *Diversity of Bacterial Respiratory Systems*, vol. 2, pp. 49–64. Edited by C. J. Knowles. Boca Raton, FL: CRC Press.

Stadtman, T. C. (1978). Selenium-dependent clostridial glycine reductase. *Methods Enzymol* **53**, 373–382.

Stadtman, T. C. & Davis, J. N. (1991). Glycine reductase protein C. Properties and characterization of its role in the reductive cleavage of Se-carboxymethyl-selenoprotein A. *J Biol Chem* **266**, 22147–22153.

Wagner, M. & Andreesen, J. R. (1995). Purification and characterization of threonine dehydrogenase from *Clostridium sticklandii*. *Arch Microbiol* **163**, 286–290.

Williams, C. H. (1992). Lipoamide dehydrogenase, glutathione reductase, thioredoxin reductase, and mercuric ion reductase – a family of flavoenzyme transhydrogenases. In *Chemistry and Biochemistry of Flavoenzymes*, vol. 3, pp. 121–131. Edited by F. Müller. Boca Raton, FL: CRC Press.

Zindel, U., Freudenberg, W., Rieth, M., Andreesen, J. R., Schnell, J. & Widdel, F. (1988). *Eubacterium acidaminophilum* sp. nov., a versatile amino acid-degrading anaerobe producing or utilizing H₂ or formate. Description and enzymatic studies. *Arch Microbiol* **150**, 254–266.

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