Design and Applications of Sensitive Enzyme Immunoassays Specific for Clostridial Enoate Reductases

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Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Clostridia, Enoate Reductase, Immunoassay, Immunological Distance

Rabbit antisera raised against purified enoate reductase from *Clostridium tyrobutyricum* (DSM 1460) and horse-radish peroxidase-conjugated staphylococcal protein A or *anti*-rabbit immunoglobulin G, respectively, were used to develop enzyme immunoassays. Sensitivity limits of the assay are about 250 pg antigen if the enzyme immunoassays are performed on membrane filters and examined visually, and 20 pg for tests in aqueous solution with spectrophotometrical evaluation. The procedures were applied for dot and Western blots as well as colony lifts. Immunological distances between enoate reductases from different clostridia were determined and the amounts of antigen present in bacterial crude extracts were estimated.

In crude extracts of *C. thermoaceticum* a protein of approximately the size of the enoate reductase and its subunits from *C. tyrobutyricum* was immunologically detected. Gel filtration chromatography of identically pretreated crude extracts from *C. thermoaceticum* and *C. tyrobutyricum* produced immunological signals at similar molecular weights and revealed a lesser tendency of the presumed thermophilic enoate reductase from *C. thermoaceticum* to disintegrate into its subunits and fragments as compared to its mesophilic counterpart.

Introduction

Enoate reductase (EC 1.3.1.31) catalyzes the NADH-dependent reduction of the carbon-carbon double bond of non-activated α,β-unsaturated carboxylates and aldehydes [1, 2]. The enzyme from *C. tyrobutyricum* DSM 1460 (formerly called *C.* La1) was purified to homogeneity and characterized as a conjugated iron-sulphur flavoprotein containing FAD and FMN composed of presumably twelve identical subunits of MW_r *ca.* 70,000 each [3].

Enoate reductase from *C. tyrobutyricum* is a versatile catalyst for biochemical reductions leading to many chiral compounds [4] owing to the high degree of stereospecificity for branched enoates, the broad substrate spectrum and the possibility to apply artificial redox mediators with whole cells or crude extracts of *C. tyrobutyricum* as well as the purified enzyme [5, 6]. Whereas, enoate reductase levels amounting to about 1% of the total cell protein were observed in *C. tyrobutyricum* cells grown on crotonate medium, enoate reductase activity in the producer strain cannot be detected by optical tests if glucose is used as the carbon source [7]. Apart from enoate reductases in *C. tyrobutyricum* and the specialist *C. kluyveri* enoate reductase activity was observed in several saccharolytic and proteolytic clostridia. Whereas, an apparent physiological role could not be assigned to enoate reductases in the former [8], for *C. sporogenes,* a typical representative of the proteolytic clostridia, participation of enoate reductase was demonstrated in the reductive branch of a Stickland reaction for the degradation of certain hydrophobic amino acids [9, 10].

It was intended to study the expression of the regulated enoate reductase gene in *C. tyrobutyricum* and, if feasible, in other hosts. Apart from the immediate genetic engineering context [11, 12] the antisera and assay procedures described here proved to be useful for several applications. Maintenance of stock cultures of producer strains, cell growth for enoate reductase production and enzyme purification processes can be routinely supervised by enzyme immunoassays that exceed the sensitivity of enoate reductase activity determinations. Furthermore, enoate reductase from different sources could be

Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen; ELISA, enzyme-linked immunosorbent assay; ER, enoate reductase; FPLC, fast protein liquid chromatography; Ig, immunoglobulin; MW, molecular weight; SDS, sodium dodecylsulphate; TBS, tris-buffered saline.

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compared immunologically, studying bacterial crude extracts without prior purification. The immunological relationship between enoate reductases from different clostridia established by Giesel and Simon [13], using immunological distance values obtained by microcomplement fixation assays, was confirmed. The practicability of the immunoassays, consuming comparatively little antiserum, allowed to extend immunological studies to a greater range of species. Looking for enzymes related to enoate reductase from *C. tyrobutyricum* in different bacterial species, binding of *anti*-enoate-reductase sera to a polypeptide of *C. thermoaceticum* was observed. The immunological evidence for the presence of enoate reductase in this thermophilic, saccharolytic organism explains the reduction of *E*-2-methylbutenoate to 2-methylbutyrate and *E*-2-methylcinnamate to 2-methyl-3-phenylpropionate at the expense of reducing agents such as hydrogen, carbon monoxide or formate by *C. thermoaceticum* [14].

Materials and Methods

Bacterial strains and growth media

Sources of the clostridial strains are listed in Table II in the Results section. If not mentioned otherwise *C. tyrobutyricum* is always the strain DSM 1460. Anaerobic cultivation of the organisms was performed in the media and under the conditions recommended by the German Collection of Microorganisms (DSM D-3300 Braunschweig). Most clostridial strains were routinely maintained in Reinforced Clostridial Medium (RCM). For enoate reductase production *C. tyrobutyricum* was grown on crotonate medium [15] and *C. sporogenes* on phenylalanine medium [10]. For growth of *C. thermoaceticum* at 60 °C the medium of Ljungdahl and Andreesen [16], was used.

To obtain protein extracts of bacteria, one part of wet packed cells was incubated for about 20 min at 37 °C in two parts of 20 mM Tris-HCl, pH 8.0; 0.9% NaCl, 1 mg/ml lysozyme and 0.1 mg/ml DNase I.

General procedures

The protein content was determined by the modified Bradford reaction [17] and enoate reductase activity as described by Bühler and Simon [18], mostly using *E*-2-methylbutenoate and electrochemically reduced methylviologen as the substrates [4]. Purified enoate reductase from *C. tyrobutyricum* was prepared as described by Kuno *et al.* [3]. Gel filtration was performed on a Superose 12 HR 10/30 column with the FPLC equipment from Pharmacia, D-7800 Freiburg. The following proteins with their molecular weights indicated in brackets were used to calibrate the gel filtration column: Aldolase (158,000), glucose-6-phosphate-kinase (84,000), albumin (67,000), ovalbumin (43,000) and chymotrypsinogen A (25,000). For immunoblotting 200 µg protein per lane were separated on 10% discontinuous polyacrylamide slab gels containing 0.1% SDS [19]. Electrotransfer of proteins to BA-85 nitrocellulose sheets with a pore diameter of 0.45 µm (Schleicher und Schüll, D-3354 Dassel) was performed in 25 mM Tris, 192 mM glycine containing 20% methanol according to Towbin *et al.* [20]. Proteins remaining in the gel were stained with Coomassie Brilliant Blue R 250 (Serva, D-6900 Heidelberg) for comparison; those transferred to the filter were visualized by Ponceau S (Sigma, D-8024 Deisenhofen). The red dye is reversibly bound to proteins and fades away at incubation in high ionic strength buffers during subsequent immunological procedures.

Immunization scheme

Eight weeks old rabbits were immunized three times in four weeks intervals by subcutaneous injection of 1.5 mg of purified enoate reductase mixed with 0.5 ml of complete Freund's adjuvant. Four weeks after the booster immunization, when specific antibodies in blood samples taken from the ear vein had been confirmed by either double diffusion [21], or immunoprecipitation [22] or test on microtiter plates (see below), the rabbits were bled and the crude sera separated from the precipitate. If necessary, antisera were preincubated with bacterial crude extracts immobilized on nitrocellulose, to remove unspecific antibodies. Part of one batch of polyclonal antiserum was subjected to ammonium sulphate precipitation.

Immunoreagents

Staphylococcal protein A conjugated to horse-radish peroxidase was purchased from Bio-Rad (D-8000 München) and horse-radish peroxidase *anti*-rabbit IgG from Sigma. Immunoreactions were performed in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl), which is the basis for TTBS that contained 0.05% Tween 20 (Bio-Rad) in TBS for block-

ing solution (3% gelatin in TBS) and for antibody buffer (1% gelatin in TTBS).

Horse-radish peroxidase stains in aqueous solutions were performed in microtiter plates made of polystyrene (C. A. Greiner und Söhne, D-7470 Nürtingen) according to Gallati and Brodbeck [23], using 15 mM phenylenediamine (Merck, D-6100 Darmstadt) as the chromogenic substrate in 100 mM citrate buffer pH 5.0 containing 5 mM H_2O_2. The staining solutions for immunodetection on nitrocellulose filters either contained 0.08% *o*-dianisidin, 0.02% sodium-nitrosyl pentacyanoferrate III and 5 mM H_2O_2, or the colour development reagent containing 4-chloro-naphthol from Bio-Rad following the instructions of the supplier.

Enzyme immunoassays

In enzyme immunoassays antigen was applied to nitrocellulose filters (Schleicher and Schüll) either by spotting and air drying protein samples onto test strips [24], or by electrotransfer of proteins from polyacrylamide gels [20], or by colony lifts [25]. For the latter assay method filter disks were positioned on the surface of nutrient agar plates, inoculated and incubated. When colonies were visible the filter disks were peeled off the agar surface and placed in a chloroform vapor chamber to kill bacterial cells and facilitate lysis. To accomplish lysis of adhering cells the filter disks were incubated overnight at room temperature in 50 mM Tris-HCl, pH 7.5; 0.15 M NaCl, 5 mM $MgCl_2$, 3% bovine serum albumin, 1 μg/ml DNase and 40 μg/ml lysozyme.

Unspecific binding of proteins of the immunoreagents onto the filters was repressed by pretreating the filters with the above mentioned blocking solution for 1 h. The antibody and protein A solutions contained 1% gelatin and 0.05% Tween 20 for the same purpose. Before overnight incubation of the filters with antisera diluted in the range 1:20 to 1:100 they were washed with TBS containing 0.05% Tween 20. This washing was repeated before adding the peroxidase-protein A conjugate at a dilution of 1:10,000 or *anti*-rabbit IgG at a dilution of 1:1000, respectively to the filters.

The wells of microtiter plates were treated correspondingly with 100 μl volumes each of the aforementioned solutions.

Because antigen adsorption to polystyrene is optimal at a protein concentration of about 1 mg/ml [26] crude extracts were diluted to this concentration.

The peroxidase reaction in 200 μl diphenylamine staining solution was stopped by adding 50 μl of 8 N H_2SO_4, which resulted in a colour change from yellow to red. Extinction values at 440 nm were recorded by means of a Titertek Multiskan ELISA-reader (Flow Laboratories).

For the determination of immunological distances [27] serial dilutions of antigen starting from 0.5 μg/μl total protein were allowed to adsorb to the microtiter plates, reacted with different antiserum dilutions and subjected to the peroxidase protein A conjugate treatment and colour development. Extinction values were plotted against the antigen dilutions. Maxima of the extinction at 440 nm of the resulting binding curves were blotted *versus* increasing dilution ratios, corresponding to the log of the antiserum dilution factor (Fig. 2). Immunological distance values are derived from the distances between the graphs for individual antigens.

Results

Characterization of antisera

The titers of the *anti*-enoate reductase sera obtained from rabbits are listed in Table I. Ouchterlony double diffusion of *anti*-enoate reductase sera against purified enoate reductase as well as crude extracts from crotonate grown *C. tyrobutyricum* yield clear and homogeneous precipitation bands (not shown). Weaker signals in Ouchterlony double diffusion tests are also visible with crude extracts from glucose-grown *C. tyrobutyricum* DSM 1460 and other *C. tyrobutyricum* strains as antigens. The enzymatic activity of enoate reductase is not inhibited by the control

Table I. Properties of the antisera.

Antiserum preparation		Protein concentration [mg/ml]	Titer[b]
Anti-enoate-reductase sera from rabbits:			
Anti-ER-1	1984	50	1:2000
Anti-ER-2	1984	76	1:2140
Anti-ER-3	1980[a]	96	1:1050
Anti-ER-4	1980[a]	89	1:2000
γ-Globulin from *Anti*-ER-2		16	1:1150

[a] Antisera also used in *l.c.* [13].
[b] Determined by enzyme immunoassay on microtiter plates.

proteins bovine serum albumin and unspecific rabbit serum, each applied at 20 μg protein per 20 mU enoate reductase (not shown). As expected, inhibition of enoate reductase activity occurs after incubation with *anti*-enoate reductase serum. Surprisingly the same observation was made to some extent with *anti*-rabbit immunoglobulin G from goat. It exhibited strong direct binding to enoate reductase in immunoassays and, therefore, was substituted by staphylococcal protein A in later experiments.

Detection limits of enzyme immunoassays for enoate reductases

The visual detection limit of the test on filters was bound to be 250 pg enoate reductase, when the reproducible 4-chloro-naphthol stain was applied. Photometric tests in microtiter plates showed a detection limit of 20 pg antigen for calibration solutions with pure enoate reductase if an arbitrary extinction difference of more than 0.03 E_{440}-units was exceeded. Unfortunately, the determination of antigen amounts is not very accurate since the response to given enoate reductase amounts adsorbed to the polystyrene matrix varies strongly with the kind and amount of other proteins present. Judging from visual examination these drawbacks are not encountered in the filter assay, which is, however, less sensitive by one order of magnitude. The sensitivity and ease of application of the enzyme immunoassay is demonstrated by the following colony lift experiment. A mixture of *C. tyrobutyricum* and a clostridial strain, producing no enoate reductase, was plated on RCM agar which contained glucose but no crotonate. Colonies were transferred to nitrocellulose disks, lysed, and resulting filter-bound proteins were subjected to enzyme immunoassay. The glucose-grown *C. tyrobutyricum* colonies were readily distinguished from other colonies lacking enoate reductase by a clearly visible immunological response (see also Fig. 1). In *C. tyrobutyricum* grown on glucose no enoate reductase can be detected by enzymatic tests.

Immunoblotting

To prove the specificity of the *anti*-enoate reductase sera, Western blot experiments were performed using several bacterial crude extracts as antigen sources. The same pattern was observed for crude extracts from the following *C. tyrobutyricum* strains: DSM 1460 (producer strain), DSM 1/1, DSM 663,

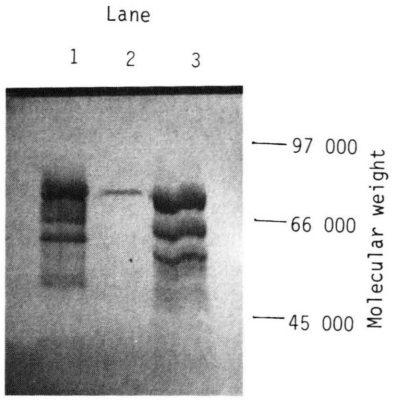

Fig. 1. Immunological comparison (peroxidase stain) of 5 μg purified enoate reductase from *C. tyrobutyricum* DSM 1460 (lane 1) and crude extract of glucose-grown *C. tyrobutyricum* (lane 2) as well as crude extract of crotonate-grown cells (lane 3). 200 μg of protein were applied to lanes 2 and 3.

and the clostridia *C. kluyveri* DSM 555, *C. sporogenes* ATCC 3584 and *C. thermoaceticum* DSM 521. The latter showed weak but, nevertheless, clearly visible signals. No signals were observed from extracts of *C. aceticum* DSM 1496, *C. acetobutylicum* ATCC 824, *C. bifermentans* ATCC 638, *C. butyricum* ATCC 19398, *C. formicoaceticum* DSM 92, *C. ghoni* ATCC 25757, *C. hastiforme* ATCC 25722, *C. pasteurianum* ATCC 6013, *C. sordellii* ATCC 9714 and *C. thermoautotrophicum* DSM 1974. Fig. 1 shows the comparison of the Western blot analysis of purified enoate reductase with that of crude extract of *C. tyrobutyricum* grown on crotonate or glucose. A single weak band can be clearly seen at 70 kDa from the crude extract of the glucose-grown cells, whereas enoate reductase cannot be detected by means of enzyme activity determinations. Since additional immunological signals arise in crude extracts from crotonate-grown *C. tyrobutyricum* and purified enoate reductase the occurrence of minor bands depends on the amount of specific antigen rather than the concentration of total protein. The patterns of lane 1 and 3 are rather similar. Slight dissimilarities in migration distances may be caused by the great differences in amounts of protein put on lane 1 and 3.

When crude extracts of other enoate reductase containing clostridia than *C. tyrobutyricum* are subjected to Western blot analysis, similar patterns of immunological signals are observed as in the case of purified enoate reductase or cell-free extracts of

C. tyrobutyricum (not shown). This indicates more or less equal molecular weights of the respective enoate reductase subunits or fragments and a rather high degree of structural similarity of the holoenzymes despite the low degree of phylogenetic relationship of the producer species and the different substrate spectra of the enzymes. In addition to species of which enoate reductase activities and their immunological relationship to enoate reductase from *C. tyrobutyricum* have been reported [10, 13], cross-reactions to *anti*-enoate reductase sera were also observed with polypeptides of *C. thermoaceticum*.

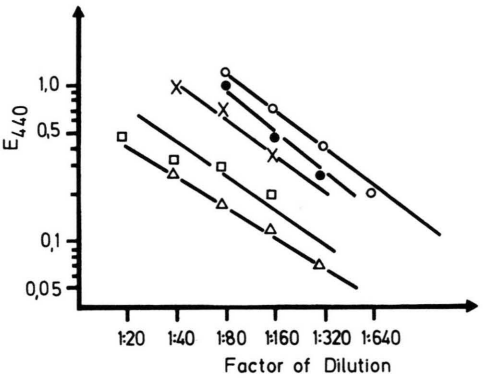

Fig. 2. Determination of immunological distances from antibody binding curves (not shown) to crude extracts. The maxima of the extinction at 444 nm of the binding curves are blotted against the increasing dilution ratios of the antiserum. *C. tyrobutyricum* DSM 1460, *C. tyrobutyricum* ATCC 25755, *C. kluyveri*, *C. thermoaceticum*, *C. sporogenes*. (For details see Materials and Methods.)

Immunological relationship of clostridial enoate reductases

After the presence of enoate reductase-like antigens in several clostridial species had been established, it was of interest to evaluate the immunological relationship between the enoate reductases of different species using the enzyme immunoassay instead of the microcomplement fixation. In order to determine immunological distances binding curves for various antigens were measured in microtiter plates (Fig. 2). It was possible to determine the immunological distances without purifying the antigenic proteins. In Table II the values are compared with those obtained by microcomplement fixation. The procedure provides rough estimations of the antigen content of cell-free extracts at the same time. Table II shows the immunologically determined enoate reductase contents of crude extracts from several clostridial strains grown under different conditions compared to the amount of enoate reductase determined by enzyme activity determinations. As already mentioned enoate reductase activity cannot be found in glucose-grown *C. tyrobutyricum* cells due to the sensitivity limits of the optical test consuming NADH. Furthermore, side reactions in *C. thermoaceticum* crude extracts render an enzymatic test of enoate reductase

Table II. Comparison of clostridial enoate reductases by tests on microtiter plates. Surveys of immunologically determined enoate-reductase contents of cell-free extracts and immunological distance values obtained by the aforementioned tests are compared with optical enzyme activity tests and microcomplement fixation data. Further explanations are given in the text. Numbers marked by an asterisk (*) are values according to enzyme enrichment data taken from Kuno *et al.* (1985). n.d. = not determined.

Antigen (crude extracts)			Enoate-reductase contents [µg/mg protein] determined		Immunological distance by	
Species	Strain	Carbon source	enzymatically mU/mg protein	immunologically µg ER/mg protein	ELISA	microcomplement fixation
C. tyrobutyricum La 1	DSM 1460	crotonate	63	60	1	1
		crotonate	11	18	1	1
		glucose	<0.3	3	1	n.d.
C. tyrobutyricum	DSM 663	glucose	<0.3	3	28 ± 5	30
C. tyrobutyricum	DSM 1/1	glucose	<0.3	2	15 ± 10	29
C. tyrobutyricum	ATCC 25755	glucose	<0.3	8	23 ± 3	n.d.
C. kluyveri	DSM 555	ethanol acetate	60	30	63 ± 30	120
C. thermoaceticum	DSM 521	glucose	n.d.	18	95 ± 15	n.d.
C. sporogenes	ATCC 3584	phenylalanine	135	1	110 ± 15	120
Purified enoate reductase from *C.* La 1			14,000*	950*	1	1

impossible. For purified enoate reductase from *C. tyrobutyricum* the ratio mU/μg protein is about 14 (last line of Table II) if the enzyme activity is determined by the optical test and the amount of protein by the Bradford procedure. Surprisingly, in crude extract of *C. tyrobutyricum* DSM 1460 this ratio is about 1–0.6 if the enzyme activity is compared to the amount of antigen determined by the test in microtiter plates. It is remarkable that nevertheless the immunologically determined amounts of enoate reductase for different species can be compared, even for those with a large immunological distance and also for protein samples derived from glucose-grown *C. tyrobutyricum* cells.

Enoate reductase-like antigens in C. thermoaceticum

Cross-reaction of *anti*-enoate reductase sera with crude extracts of *C. thermoaceticum* had been shown and the antibody binding polypeptide had turned out to be of approximately the same size of enoate reductase subunits determined for those of enoate reductase from *C. tyrobutyricum* DSM 1460. For further investigations of the enoate reductase-like antigen of *C. thermoaceticum* and its comparison with enoate reductase from *C. tyrobutyricum* crude extracts of cells of both species were stored and treated in exactly the same manner. To remove cell debris the crude extracts were subjected to centrifugation at $50{,}000 \times g$ prior to gel filtration. The experiments were carried out without the exclusion of oxygen. By gel filtration on a Superose 12 column native proteins of the crude extracts from *C. tyrobutyricum* and *C. thermoaceticum* were separated according to their molecular weights. The protein concentrations of the collected fractions and corresponding enzyme immunoassays in microtiter plates after treatment with *anti*-enoate reductase serum were recorded (Fig. 3). It is surprising that in the presence of oxygen almost no native enoate reductase of a relative molecular weight of 920,000 can be observed. The main peak appears at MW_r of about 250,000. Fractions with MW_r as low as 25,000 can be observed. This seems to be related to the phenomenon shown in Fig. 1. Most of the enoate reductase-like antigen from *C. thermoaceticum* reveals a MW_r of about 1 million.

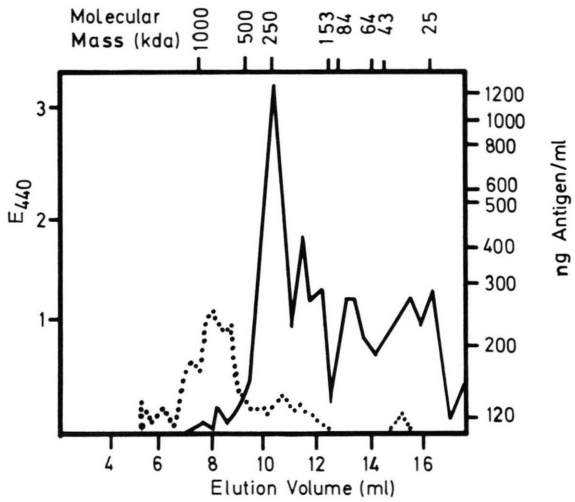

Fig. 3. Gel filtration of crude extracts from *C. tyrobutyricum* DSM 1460 (solid line) and *C. thermoaceticum* (dotted line) on FPLC Superose 12 with subsequent immunological detection of enoate reductase. By calibration of the column with standard proteins the molecular weights of eluted proteins were estimated.

Discussion

Specificity and sensitivity of immunological enoate-reductase assays

Using purified enoate reductase for calibration as little as 250 pg enoate reductase from *C. tyrobutyricum* on nitrocellulose and 20 pg on microtiter plates could be detected. Thus, the assays exceed by far the sensitivity of enoate-reductase activity determinations following the reoxidation of NADH or reduced methylviologen photometrically. At least 500,000 pg of active enzyme is required to produce signals by this method. Amounts of enoate reductase not longer detectable by enzyme assays can be detected in colony lift experiments, on nitrocellulose filters and in single colonies of glucose-grown *C. tyrobutyricum*, where enoate-reductase formation is suppressed. The combined application of colony lifts from RCM-plates and immunoassays greatly facilitates monitoring stock cultures for enoate-reductase production. Sufficient lysis of clostridial colonies occurs in the immunoassay experiments, whereas, the failure of many gram-positive bacteria to lyse properly on filter disks constitutes a major problem for the application of colony hybridization and immunoassay techniques to this taxonomic group. The sensitivity of the non-radioactive assays seems sufficient to demonstrate heterologous expression of genetically engineered enoate reductase gene.

Table II shows attempts to quantify immunologically the enoate reductase contents of bacterial lysates. The *anti*-enoate reductase sera and assay methods may allow the elucidation of the regulation of enoate-reductase expression.

Surprising is the fact, that in crude extracts the amount of proteins acting as antigens seems to be much higher than enzymatically active protein.

The most critical test for the specificity of antisera and immunoassays is immunoblotting of electrophoretic separations of complex protein mixtures containing the antigen. Generally, one sharp band appears, when highly specific antibody preparations are used, and additional signals indicate unspecific binding [28]. The Western blot patterns in Fig. 1 show similar patterns for SDS-PAGE electrophoresis of crude extract of *C. tyrobutyricum* grown on crotonate and purified enoate reductase. A faint, but nevertheless clear band is also seen from crude extract of glucose-grown *C. tyrobutyricum* at a molecular weight of about 70,000. Under native conditions in gel filtration separation experiments immunological signals centered around of MW of 35,000 account for 20% of the antigen in *C. tyrobutyricum* and for 5% in *C. thermoaceticum* (Fig. 3). This behaviour differs significantly with results of ultracentrifugation or gel filtration experiments under strict exclusion of oxygen [3]. It seems that oxygen causes the formation of fragments not identical with the formerly determined subunits [3].

However, one has to keep in mind that there exist difficulties in quantification of enoate reductase in microtiter plates probably due to odd binding characteristics of the antigenic protein with an isoelectric point of 8.4 [1] to the polystyrene matrix. These were not encountered to the same degree by visual examination of the less sensitive tests on filter disks.

Determination of immunological distances

So far immunological comparisons of related proteins mainly have been conducted by microcomplement fixation assays [27]. We found that in the case of enoate reductase the sensitivity of the easy to perform enzyme immunoassays is sufficient to establish at least rough immunological distance values with small amounts of antisera. The results of the two independent methods are in reasonable agreement (Table II). Neither bacterial lysates containing the antigens, nor the crude antisera needed any purification or enrichment steps. The test in microtiter plates omits errors owing to anticomplementarity occasionally occurring in microcomplement fixation assays. Observations on lysozyme suggest that five immunological distance units correspond to 1% amino acid deviation [29]. The enoate reductases from *C. sporogenes* and *C. thermoaceticum* are thus expected to share about 80% overall amino acid sequence homology with the enzyme from *C. tyrobutyricum*.

Concerning immunological distance values generally immunological cross-reaction is not expected, if the divergence in the amino acid composition is greater than 30%, which corresponds to immunological distance values above 150 [30].

Enoate reductase-like antigen in C. thermoaceticum

The occurrence of an enoate reductase in *C. thermoaceticum* comes as a surprise, as close phylogenetic relationship between *C. tyrobutyricum* and *C. thermoaceticum* are improbable because of the very different G + C content of their DNA of 50% for the former and 30% for the latter [31]. Enzymatic enoate-reductase determinations in crude extracts of *C. thermoaceticum* are prevented by the occurrence of overlaying reactions using NADH or reduced viologens. Products of preparative hydrogenations of enoates using whole cells of *C. thermoaceticum* show, however, the stereospecificity typical for the reduction of non-activated α,β-unsaturated carboxylates by enoate reductases [14]. The double bond in α,β-unsaturated CoA esters usually is hydrogenated in a *trans*-fashion opposite to the stereochemical course of enoate-reductase catalysis [32].

One should also mention the fact that *C. formicoaceticum*, which is very similar to *C. thermoaceticum* [33], seemed not to have an enoate reductase-like antigen, although *E*-2-methylbutenoate is reduced to 2-methylbutyrate by *C. formicoaceticum* at the expense of carbon monoxide [34]. However, according to preliminary results the stereochemical course of this reduction does not lead to (2*R*)-methylbutyrate but to the (2*S*) enantiomer (unpublished).

In a quantitative enzyme immunoassay conducted on microtiter plates following gel filtration of identically pretreated crude extracts of *C. tyrobutyricum* and *C. thermoaceticum* the stability of native enoate

reductases turned out to be different. Comparison of immunological signals at the molecular weights corresponding to differently sized subunit aggregates reveals that the holoenzyme from *C. tyrobutyricum* more readily disintegrates into its subunits and apparent fragments than the suspected homologous enzyme from *C. thermoaceticum*.

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