Limited proteolysis of lactate dehydrogenase from porcine heart with trypsin: Characterization and reactivation of the fragments

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Summary. The ternary complex formed by native lactate dehydrogenase (LDH) from porcine heart, NAD^+ and sulfite, was digested with trypsin over a period of 12–16 h^3. After removal of the ligands and residual native lactate dehydrogenase by ion exchange chromatography dimers were obtained which were almost inactive. The dimers were lacking a hexapeptide at the N-terminus; however, the secondary structure was the same as that of native lactate dehydrogenase. The circular dichroism spectra showed a dependence on temperature which suggested an equilibrium of two different structural states.

The reaction of antibodies against native porcine heart LDH with the dimers restored the catalytic activity, and subsequently the dimers behaved similarly to the native enzyme. Addition of 1 M phosphate or NAD-sulfite to the dimers restored 80–90% of the catalytic activity. It could be demonstrated that the behaviour of the reactivated dimers, in contrast to that of the inactive dimers, was similar to the behaviour of native lactate dehydrogenase. For instance, ultracentrifugal analysis showed that dimers reactivated with NAD-SO_3^- were associated to give tetramers. The reaction of antibodies against native LDH with the dimers reactivated with NAD-SO_3^- demonstrated that the native LDH and the dimers have the same surface determinants.

Key words. Lactate dehydrogenase from porcine heart; limited proteolysis of LDH; proteolytic dimers; reactivation and association; antibodies against LDH; temperature dependence of CD-spectra; high/low-temperature state of the dimers; structural relations.

The most important difference between prokaryotic and eukaryotic lactate dehydrogenase (LDH) is found in the N-terminal part of the protein. Bacterial LDH lacks the first 14 residues which build the N-terminal arm in the vertebrate enzyme. Although the residual structures of bacterial and vertebrate LDH are homologous, bacterial LDH exists as a dimer. Fructose-1,6-diphosphate activates the bacterial dimers, which associate to form a tetramer[1]. Therefore, it was interesting to find out whether, after removal of the N-terminal arm, eukaryotic LDH would show behaviour similar to that of prokaryotic LDH with respect to activity and association states. Lactate dehydrogenase from porcine heart is a tetramer composed of four equal subunits. On detailed consideration it is clear that the tetrameric state has to be described as a 'dimer of dimers' stabilized by the N-terminal arm of 20 amino acid residues[2]. The interactions between the subunits are divided into 3 main contacts (P,Q,R) referring to an orthogonal system, where the N-terminal residues are entirely related to the R-contact. Limited proteolysis with a highly specific protease such as trypsin is an important tool for studying the topography of protein surfaces. The tryptic digestion of the ternary complex of porcine heart LDH with NAD^+ and sulfite was described previously[3]. The activity of the proteolytic fragments obtained depends on the presence of NAD^+ and sulfite. In the presence of the ligands the activity of the digested LDH increased from 2% up to 90%. In this report, no further explanations for this behavior were given[3]. Therefore a closer inspection of this limited proteolysis and the resulting fragments seemed likely to be interesting. Previous investigations[4] demonstrated that thermolysin treatment of porcine muscle LDH during renaturation yields proteolytic dimers. In the present report, tryptic digestion of native LDH from porcine heart, and the characterization of the resulting fragments, is described.

Materials and methods

General chemical. The porcine H_4 isoenzyme of LDH was isolated from porcine heart as described below. NADH and NAD^+ were obtained from Boehringer, Mannheim; Sepharose Cl 4B and Sephadex G-25 were from Pharmacia, Uppsala. TPCK-treated trypsin was purchased from Sigma Chemical Co., St. Louis, MO. Trypsin inhibitor (soybean) and Coomassie Brilliant Blue G-250 were obtained from Serva, Heidelberg. Fractogel DEAE 650 and Blue Fractogel are products of Merck, Darmstadt, and DEAE-cellulose DE 52 was from Whatman, Maidstone. The antibodies were a gift from Merck, Darmstadt and were purified as described below. Cellulose sheets Polygram CEL 300 for high-voltage electrophoresis are products of Machery & Nagel, Düren. Buffer substances and all other reagents were of analytical grade or the best grade available. All buffer solutions were degassed and 0.02% sodium azide was added.

Enzyme purification. The LDH and other proteins were extracted from the crude homogenate by ion exchange (DEAE-cellulose) at pH 7.2. The proteins were detached with 100 mM phosphate buffer pH 7.2 and the LDH was purified by affinity chromatography. The ligand used in

affinity chromatography was the triazine dye color cibacrone blue[5]. Washing with standard buffer removed undesired proteins. A 10 mM solution of NAD^+ in standard buffer yielded the purified native porcine heart LDH. NAD^+ was removed by gel chromatography (G-25). The fractions with high activity were pooled and had a specific activity of 420 IU/mg. No hybrid could be detected by acetate sheet electrophoresis. The native LDH was precipitated by adding solid ammonium sulfate to a final saturation of 65%, and stored in suspension at 4°C.

Enzyme assays. Enzyme activity was determined in 0.067 M phosphate buffer pH 7.2 (standard buffer) containing 0.3 mM NADH and 0.5 mM pyruvate at 25°C. The oxidation of NADH was monitored at 366 nm in a 1-cm cuvette using an Eppendorf photometer fitted with a thermostatically controlled cuvette holder. Enzyme concentrations of native LDH and digested LDH were determined with a Jasco UVIDEC-610 spectrophotometer on the basis of $A^{0.1\%}_{280\,nm} = 1.38$. Application of the same absorbance factor for the native and the digested enzyme is permitted because the absorbance change at 595 nm caused by the binding of Coomassie Brillant Blue G-250[6] to the protein is equal for the native and the digested enzyme. In the presence of NAD^+, enzyme concentrations were also determined from the absorbance change at 595 nm caused by Coomassie Brillant Blue G-250, using native LDH as standard.

Proteolysis. The proteolysis was carried out at 25°C in 50 mM tris buffer pH 8.0 containing 10 mM $CaCl_2$, 3.7 mM NAD^+, 3.9 mM Na_2SO_3, 5 mg/ml LDH and 0.05 mg/ml trypsin (protein/protease ratio 100:1). After 12–16 h the digestion was stopped by the addition of trypsin inhibitor from soybean to a final concentration of 0.15 mg/ml. To remove Ca^{2+} ions and to concentrate the protein a centricon-30 (Amicon, Danvers) was used. The digestion mixture was introduced onto a column (1.5 × 8.0 cm) packed with Fractogel DEAE 650. Washing thoroughly with 10 mM phosphate buffer pH 7.0 containing 50 mM NaCl, using a peristaltic pump (Colora), removed bound $NAD\text{-}SO_3^-$. Elution with a gradient of 0.1 – 0.2 M sodium chloride in 10 mM phosphate buffer pH 7.0 separated the digested enzyme from the remaining uncleaved native LDH and the inhibited trypsin (elution profile, fig. 1).

Perchlorid acid precipitation. To separate the low-molecular-weight peptides from the digestion mixture proteolysis was carried out in 0.05 M ammonium hydrogen carbonate buffer. The protein was precipitated by slowly adding perchloric acid to a final concentration of 0.5 M. The precipitated proteins were removed by centrifugation. Neutralization of the remaining solution with KOH caused precipitation of $KClO_4$, which was washed with a small amount of cold water. After centrifugation the solution was lyophilized.

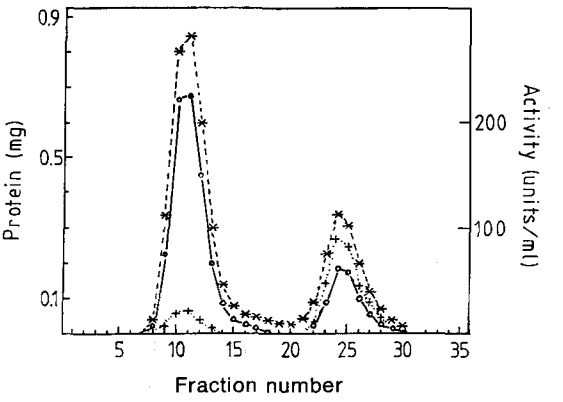

Figure 1. Ion exchange chromatography of the partially digested LDH (sample buffer 50 mM NaCl in 10 mM phosphate buffer pH 7.0; column volume 15 ml; flow rate 1–2 ml/min; fraction size 2 ml; 25°C). Elution with a gradient of 0.1–0.2 M NaCl in 10 mM phosphate buffer pH 7.0 –∗–, protein concentration; · + · · + · ·, enzymatic activity; –o–, enzymatic activity in the presence of $NAD\text{-}SO_3^-$.

High voltage paper electrophoreses. High-voltage electrophoreses were performed in a Perograph 64 (Vetter, Germany) using cellulose sheets and an acetic acid/pyridine/water buffer pH 6.0.

Amino acid and end group analysis. Amino acid analysis of the split peptides were performed in a Biotronic amino acid analyzer LC 6000 A after 24 h hydrolysis at 110°C in 6 N HCl[7]. End group analysis by dansylchloride and carboxypeptidase A was carried out using standard techniques[8]. The N-terminal sequence of the truncated enzyme was obtained from a gas phase sequencer.

Ultracentrifugation. Estimation of molecular weight was carried out in a saccharose density gradient using a Beckman ultracentrifuge L5-50 fitted with a rotor SW 60 TI. A maximum of 100 µl of the sample, with a protein concentration of 1.5 mg/ml, was layered onto a 4-ml gradient of 5–18% of sucrose in the buffer required. After centrifuging for 18 h at 45 000 rpm the gradient was fractionated into 30 fractions using a fraction collector (LKB, Bromma). Enzymatic activity and the protein concentration of each fraction were determined as described above.

Reactivations. Enzymatic activities of reactivated LDH_{tr} are related to those of analogously-treated native LDH. The ratio of protein and $NAD\text{-}SO_3^-$ for the reactivation of the LDH_{tr} is the same as in the digestion mixture. On account of the strong inhibition of the enzymatic activity by sulfite[9] higher concentrations of $NAD\text{-}SO_3^-$ are not usable. The protein solutions were prediluted in standard buffer.

Formation of the ternary complex. The formation of the ternary complex was started by adding a sulfite solution of the solution containing the protein and NAD^+. The concentrations of the three compounds were: LDH (in subunits) 0.73×10^{-4} M; NAD^+ 1.8×10^{-4} M and sulfite 2.3×10^{-4} M. The kinetics were recorded in a Jasco

UVIDEC-610 spectrophotometer using the fixed wavelength mode at 320 nm. Steaming-up of the cuvettes at 0 °C was prevented by a gentle stream of nitrogen.

Immunological experiments. The antiserum against native porcine heart LDH was purified by affinity chromatography using a protein A – sepharose CL 4B – column. 2 ml of the antiserum in tris/HCl-buffer pH 9.5 were introduced onto the protein A – sepharose CL 4B column, and incubated for 1 h. Afterwards, the column was washed with the tris/HCl buffer and the IgG-fractions were eluted using a 0.1 M glycine/HCl-buffer pH 3.0. The IgG fractions were collected in 0.3 M phosphate buffer pH 7.2 and dialyzed against standard buffer. The solution obtained was concentrated to the original volume. The titer of the IgG-solution was 290 units/ml.
An immune titration technique was applied to correlate the immunological reactivity and the activity, i.e. the percentage reactivation of the native enzyme and the truncated enzyme. Increasing amounts of antibodies were added to aliquots of enzyme solution. After incubation for 1 h at 37 °C and storage at 4 °C for 12 h the samples were centrifuged. Afterwards, the remaining activity of each sample was determined and related to the 100% value of the native LDH.

Circular dichroism. CD spectra were recorded in a Jasco 500 A spectrometer connected with a data processor 500 N.

Fluorescence spectroscopy and light scattering. Intrinsic protein fluorescence and light scattering were recorded in a Spex Fluorolog 2.

Fluorescence spectroscopy:	excitation at 290 nm emission from 280 nm to 450 nm
Light scattering:	excitation of 400 nm emission at 400 nm

Results and discussion

A) Limited proteolysis. On considering the sequence of the native LDH, with regard to the trypsin specificity for lysine and arginine, several cleavage points could be expected. The sequence of the C- and N-terminal residues[10] as well as the possible cleavage points are given in table 1. The proteolytic fragment could be separated from residual native LDH by ion exchange chromatography, which indicated that the surface charge had changed. Figure 1 shows the protein concentration of the fractions collected by ion exchange chromatography and the catalytic activity in the presence and in the absence of NAD-SO_3^-. The digested enzyme (fractions 8–17) is reactivated by adding NAD-SO_3^-, whereas native LDH (fractions 22–30) is inhibited by sulfite. About 50–60% of the starting protein amount could be recovered as proteolytic fragments.
The present conception of the function of the N-terminal arm in the quaternary structure of native mammalian LDH H_4 is that it stabilizes the tetrameric state[2]. For example the structurally closely related mitochondrial malate dehydrogenase lacks this N-terminal arm and forms stable dimers[11]. Limited proteolysis during the reconstitution of the acid dissociated LDH from porcine muscle yields stable dimers, lacking 10–11 amino acid residues at the N-terminus but presenting a heterogenous shortened C-terminus[4].
The structure of the active ternary complex of porcine LDH H_4[12] has to be envisaged as a globular structure with an exposed N-terminal arm. Therefore an attack on the ternary NAD-sulfite LDH complex by trypsin should occur preferentially on the N-terminal arm. The splitting-off of a hexapeptide at the N-terminus, described in the present investigation, causes the dissociation of the tetramer under physiological conditions into nearly inactive dimers. No other method has been found to produce homogenous LDH dimers which are stable at 0 °C for about 12 h and are accessible to biochemical and physical investigation.

B) Detection of the fragments. After perchloric acid precipitation of the protein, the low-molecular-weight peptides remaining in the supernatant solution were separated by high voltage electrophoresis. Comparing the amino acid composition of the two peptides obtained (1st peptide: 1 Ala, 1 Glu, 1 Leu, 2 Lys, 1 Thr; 2nd peptide: 1 Ala, 1 Glu, 2 Leu, 3–4 Lys, 1 Thr) with the sequence in

Table 1. *a* N- and C-terminal amino acid sequences of LDH including the possible cleavage points ot trypsin(↓); *b* sequence of the truncated LDH determined by gas phase sequencer (italics).

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                    ↓5      ↓
Ac–Ala–Thr–Leu–Lys–Glu–Lys–Leu–Ile–Ala–Pro–Val–Ala–Gln–Gln–
  15                20      ↓
–Glu–Thr–Thr–Ile–Pro–Asn–Asx–Lys–Ile–Thr–Val–.....
                    325     ↓  330  ↓
....–Asp–Thr–Leu–Trp–Gly–Ile–Gln–Lys–Asp–Leu–Lys–Asp–Leu
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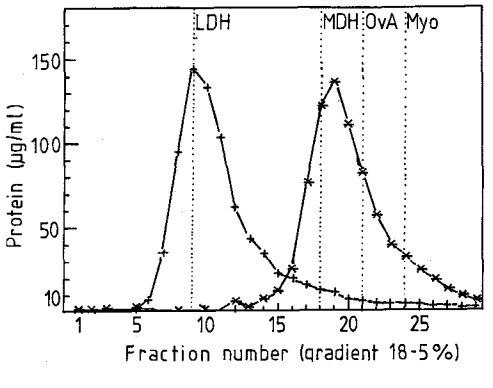

Figure 2. Determination of the molecular weights of the native LDH and the digested LDH with a saccharose gradient (18%-5%) by ultracentrifugation (concentration of the protein solutions: 1.5 mg/ml; incubation volume 100 µl; only the main fraction of the calibration enzymes is noted; calibration enzymes are: lactate dehydrogenase (LDH) 140,000, malate dehydrogenase (MDH) 68,000, ovalbumin (OvA) 45,000 and myoglobin (Myo) 17,800; rotor temperature 5 °C).
–*–, LDH_{tr}; – + –, LDH_{tr} + NAD-SO_3^-.

table 1 suggests the splitting-off of a hexapeptide at the N-terminus. Peptide 2 is probably a mixture of peptide 1 and a peptide cleaved after ^{4}Lys. The assumption that a hexapeptide is digested off is corroborated by N-terminal dansylation of the digested LDH, which yields leucine. Identification of the N-terminal sequence of the proteolytic fragments using a gas phase sequencer (table 1) also produced evidence for the digestion of a hexapeptide. As would be expected C-terminal proteolysis with carboxypeptidase A showed no difference between native LDH and truncated LDH.

The molecular weights of the LDH fractions resulting from ion exchange chromatography were determined, by density gradient centrifugation, to be 140 000 for the native tetrameric LDH, 60 000 for the proteolyzed LDH, and 140 000 for the proteolyzed LDH in the presence of NAD-SO_3^- (fig. 2).

C) Reactivation of the dimers by NAD^+ and SO_3^{2-}. The specific activity of the dimers is 2–3% of that of the native LDH, measured under standard conditions. After the addition of 1 mM NAD^+ and 1 mM sulfite the activity increased up to 90%. Besides reactivation, the formation of the ternary complex LDH-NAD-sulfite could be observed by the characteristic absorption at 320 nm. The native LDH forms the complex instantaneously, so that neither at 25 °C nor at 0 °C can the kinetics of process be measured. In contrast to this, the formation of the ternary complex of LDH_{tr} in 67 mM phosphate buffer pH 7.2 at 0 °C could be measured over a period of 20 min as a first order reaction (fig. 3). The formation of the tetrameric state in this case (see section D) also demonstrates that a structural relationship between the active site and subunit binding regions, such as a P-contact has to be supposed. The rearrangement of the P-contact must involve the active site. On the other hand the binary complex with NADH causes no significant reactivation and no association.

D) Reactivation of the dimers by anions. Another mechanism has to be postulated in the presence of high phosphate concentrations. 1 M phosphate buffer pH 7.2 at 25 °C effects about 90%–94% reactivation of the LDH_{tr} within 3 min. Further attempts to reactivate LDH_{tr} by adding other anions or ligands are collected in table 2. If LDH_{tr} is reactivated in 1 M phosphate buffer, the formation of the ternary complex takes place at 25 °C immediately, as in the case of native LDH.

Table 2. Reactivation of LDH_{tr} by different ligands (native LDH = 100%)

Ligand	Concentration	% reactivation
NAD-$SO_3^{\ominus}$	1×10^{-3} M	90
Fru-1,6-P_2	$2\text{-}20 \times 10^{-3}$ M	1.5
NADH	5×10^{-3} M	5
NADH-oxamate	2×10^{-3} M	48
NaCl	1 M	5
$(NH_4)_2SO_4$	1 M	65
Phosphate	1 M	95
Citrate	1 M	8

The time-dependence of the reactivation by phosphate ions implies also that in the course of the reactivation the dimers associate to tetramers. The association is probably caused by the weak binding of the phosphate to the anion binding site of the subunit. Therefore the binding of phosphate might be compared with the binding of fru-1, 6-P2 to bacterial LDH, which also results in an association of the dimers accompanied by activation[1]. Considering the structural properties of the anion binding site and its surroundings, the connection of the active site and the anion binding site becomes evident. Figure 4 shows a possible connection of the binding sites. In this pattern a structural rearrangement of the anion binding site by phosphate entails also a rearrangement of the substrate binding site and the P-contact. Perhaps the reactivation and the association of the dimers that are brought about by phosphate are two simultaneous processes. First experiments with light scattering corroborate that dimeric LDH_{tr} associates to the tetrameric state in 1 M phosphate buffer.

E) Circular dichroism. The examination of circular dichroism between 180 nm and 250 nm ('backbone'-

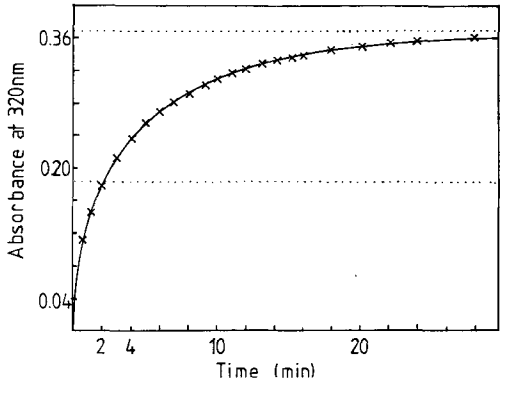

Figure 3. Absorption at 320 nm caused by the formation of the ternary complex of LDH_{tr} and NAD-SO_3^- at 0 °C (concentrations: LDH_{tr} (in subunits) 0.73×10^{-4} M; NAD^+ 1.8×10^{-4} M and SO_3^{2-} 2.3×10^{-4} M).

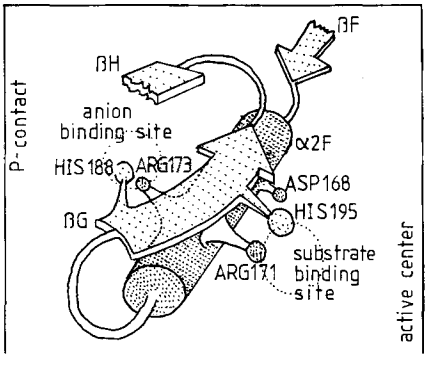

Figure 4. Diagram of the structural relationship between the active binding site and the anion binding site in the LDH.

area) yields information on the secondary structure of a protein. Only a small difference between the CD spectra of the dimers and the native enzyme was observed in this range (fig. 5). This was obviously caused by the loss of the N-terminal αA-helix. Neither at 25 °C nor at 0 °C was any temperature-dependence of the 'backbone' spectra detected.

CD spectra of the LDH_{tr} from 250 nm to 330 nm show a strong temperature dependence in the range from 0 °C to 36 °C (fig. 6). The CD spectrum of the dimers at 25 °C in standard buffer is similar to the spectrum of the native enzyme, but the signal strength seems to be reduced (fig. 7). When the spectra of the native LDH and of the LDH_{tr} at 0 °C are considered, the situation is not so simple, especially as the spectra of the dimers in 1 M phosphate buffer are no longer temperature-dependent and are identical with the native LDH CD spectrum. As proved by light scattering, no association or dissociation of the LDH_{tr} takes place in 67 mM phosphate buffer pH 7.2 at 0 °C and 25 °C. On the other hand, in the presence of 1 M phosphate buffer pH 7.2, light scattering of LDH_{tr} shows a doubling of the signal strength, which indicated an association of the dimers to the tetramers. Probably, a temperature-dependent equilibrium of two different structures of the dimeric LDH_{tr} exists in 67 mM phosphate buffer pH 7.2 and these two structures show different CD-spectra.

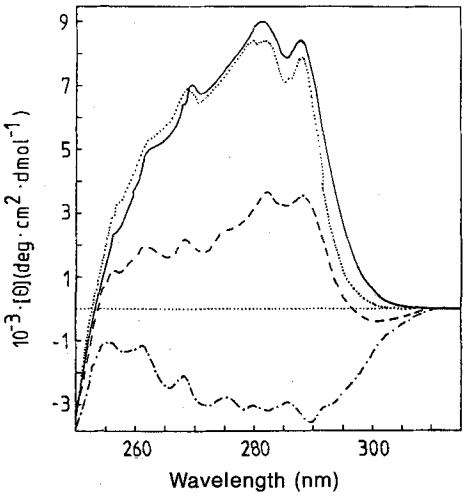

Figure 7. Circular dichroism spectra (250 nm – 330 nm) of native LDH and LDH_{tr} (protein concentration (in subunits): 2.86×10^{-6} M; optical path 10 cm).
—, native LDH (standard buffer and 1 M phosphate buffer pH 7.2; 0 °C + 25 °C); – – –, LDH_{tr} (standard buffer; 25 °C); – · –, LDH_{tr} (standard buffer; 0 °C); ·····, LDH_{tr} (1 M phosphate buffer pH 7.2; 0 °C + 25 °C).

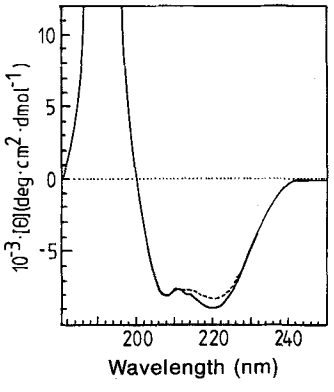

Figure 5. Circular dichroism spectra (180 nm – 250 nm) of native LDH and LDH_{tr} (protein concentration (in subunits): 15.7×10^{-6} M in standard buffer; optical path 0.1 mm; 25 °C).
—, native LDH; – – –, LDH_{tr}.

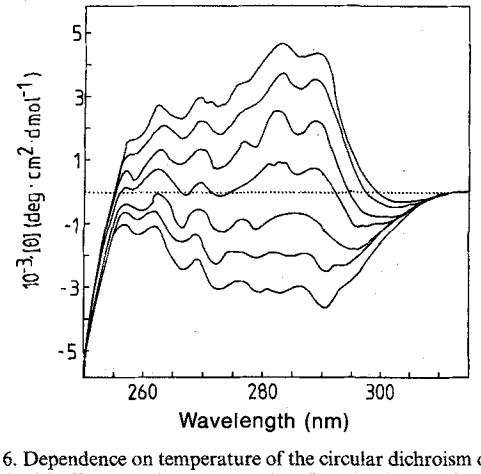

Figure 6. Dependence on temperature of the circular dichroism of LDH_{tr} in standard buffer (protein concentration (in subunits): 2.68×10^{-6} M; optical path: 10 cm) temperatures (from top to the bottom): 36, 25, 20, 14.5, 9.5, 5, 0 °C.

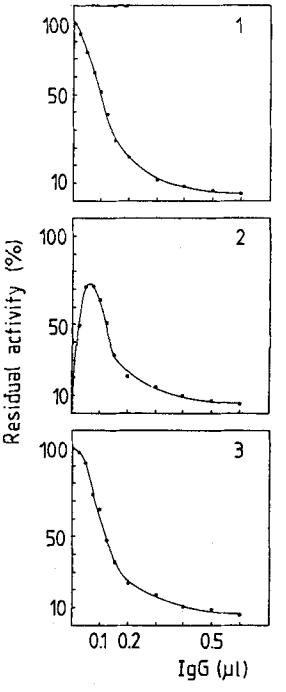

Figure 8. Immunotitration of native LDH (1), LDH_{tr} (2) and LDH_{tr} + $NAD\text{-}SO_3^-$ (3) with purified IgG from rabbit (predilution of the IgG stock solution: 1:100; amount of protein: 0.09 µg).

F) Fluorescence spectroscopy. Another method of studying structural alterations in proteins is the examination of the intrinsic fluorescence. Compared with native LDH, the maximum signal intensity at 340 nm of the LDH_{tr} is about 30% reduced.

Reactions with antibodies. Another mechanism of reactivation was observed in the presence of antibodies directed against native LDH. Antibodies react with surface determinants of a protein, which are elements of the tertiary structure. Antibodies against native LDH also react with the dimers, and this results in a reactivation of up to 70% (fig. 8, 2). Reactivation of the dimers was only detected in the supernatant solution at low concentrations of antibodies. At higher concentrations of antibodies a precipitation of antigen-antibody-complex starts, whose course is similar to that observed for the native enzyme. This result implies that the reactive surface determinants of LDH_{tr} and the native enzyme have extensive similarities. In the complex formed with low concentration of antibodies the LDH_{tr} is reactivated but not precipitated (soluble complex). This increase of activity is a result of a rearrangement of structural deviations and reassociation directed by the antibodies. Only the reactivated form of the LDH_{tr} is able to precipitate with higher concentrations of antibodies such as the native LDH. On the other hand, the ternary complex (LDH_{tr}-NAD-SO_3) and the native enzyme show similar precipitation profiles at the beginning of the reaction with antibodies, which indicated that both have the same surface determinants (fig. 8, 3 and 8, 1). This corroborates with the result that structural properties are rearranged when the ternary result that structural properties are rearranged when the ternary complex is formed or the active site is rearranged.

Abbreviations. CD, circular dichroism; DEAE, diethylaminoethyl; fru-1,6-P_2, fructose-1,6-bisphosphate; IgG, immunoglobulin G; LDH_{tr}, tryptic digested lactate dehydrogenase (isoenzyme from porcine heart) without any ligands); LDH, lactate dehydrogenase (isoenzyme from porcine heart); TPCK, N-tosyl-L-phenylalanyl-chloromethane; Tris/HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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High sensitivity of porcine cerebral arteries to endothelin

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Summary. The threshold concentration of endothelin to induce contractions in porcine cerebral arteries (anterior cerebral, Willis ring and basilar artery) was lower than those for coronary and renal arteries. The median effective concentrations (ED_{50}) of endothelin in cerebral arteries were also significantly lower than those for coronary and renal arteries. There was no significant difference in the sensitivity to endothelin among cerebral arteries, or between coronary and renal arteries. The maximal percentage of contractions induced by endothelin, as compared to that induced by 10^{-1} M potassium chloride, was not significantly different between the arteries.
Key words. Endothelin; porcine; cerebral artery; coronary artery; renal artery.

Large cerebral arteries play a major role in the regulation of blood flow in the brain [1]. With regard to the effects of vasoactive substances on cerebral and peripheral arterial tonus, there are larger differences between cerebral and peripheral arteries in the sensitivity to agonists [2, 3]. This heterogeneity of response to various agonists exists even among the cerebral arteries [4]. Endothelin, which has recently been isolated from endothelial cells, plays an im-