Proteolytic Dimers of Porcine Muscle Lactate Dehydrogenase: Characterization, Folding, and Reconstitution of the Truncated and Nicked Polypeptide Chain†

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ABSTRACT: Lactate dehydrogenase from porcine skeletal muscle is a "dimer of dimers" that is stabilized in its tetrameric state by an N-terminal "arm" of ~20 amino acid residues. Due to the low dissociation constant of the tetramer, the dimer is inaccessible to direct analysis. Limited proteolysis during reconstitution (after dissociation at pH 2.3) yields stable "dimers". As suggested by affinity chromatography, these inactive dimers contain the dinucleotide fold of native LDH. In the presence of structure-making ions, ~40% activity is restored in the dimeric state [Girg, R., Jaenicke, R., & Rudolph, R. (1983) *Biochem. Int. 7*, 443–444]. The cleavage yields about equal amounts of three fragments, F 34, F 21, and F 14 (M_r 33.5K, 21.4K, and 13.5K, respectively). F 34 represents the intact chain lacking the N-terminal 10–11 amino acid residues; its C-terminus is heterogeneous, varying in the range between residues 326 ± 5. F 21 contains residues 11/12 to 200 ± 3; F 14 is a mixture of three subfragments: residues 11/12 to ~133, 38 to ~163, and 208 to ~327. After solubilization in 6 M guanidine hydrochloride, F 34 can be reconstituted to partially active dimers. Reactivation is determined by slow subunit refolding with subsequent diffusion-controlled dimerization, in accordance with the monomer–dimer transition in the reconstitution mechanism of the intact tetramer. Reconstitution of F 21 and F 14 is concentration dependent and leads to partially active "nicked dimers", indicating that separate domains are able to reassociate correctly to yield the native subunit arrangement.

Limited proteolysis has been a useful tool to characterize conformational states and conformational changes in monomeric and oligomeric proteins (Rupley, 1967; Gervais et al., 1980; Goldberg & Zetina, 1980; Kirschner & Szadkowski, 1980; Girg et al., 1981; Zettlmeissl et al., 1983; Rudolph, 1985). In connection with the in vitro folding of proteins, the method has been successfully applied to monitor single steps in the folding pathway of ribonuclease (Schmid & Blaschek, 1984; Lang & Schmid, 1986) and octopin dehydrogenase (Teschner et al., 1987) and in the assembly of the domains of the β_2 subunit of tryptophan synthase (Goldberg & Zetina, 1980).

In the case of the reconstitution of porcine muscle lactate dehydrogenase from its denatured polypeptide chains, Girg et al. (1981) were able to show that thermolysin treatment during renaturation yields "proteolytic dimers" as stable entities. A closer inspection of the reaction product by SDS[1]–polyacrylamide gel electrophoresis indicated that the "dimers" consist of virtually intact subunits lacking only a small portion of their polypeptide chain. In addition, "nicked" smaller fragments have been observed.

Evidence from spectral and hydrodynamic measurements, as well as binding to a dinucleotide specific affinity column (Procion Green), proved the backbone structure of the truncated dimers to be closely similar to the native tetramer. The observation that in the presence of stabilizing salt [e.g., $(NH_4)_2SO_4$] the proteolytic dimers exhibit catalytic activity corroborates this finding (Girg et al., 1983a). Previous attempts to achieve homogeneous fragments failed (Girg et al., 1983a). Therefore, no unambiguous characterization of the fragments was possible. In this investigation a full account of the cleavage pattern at varying conditions of proteolysis is presented together with a detailed characterization of the fragments and their denaturation–renaturation behavior.

Materials and Methods

Materials. Porcine skeletal muscle lactate dehydrogenase and NADH were obtained from Boehringer, Mannheim; thermolysin (*Bacillus thermoproteolyticus*) and carboxypeptidase Y were from Serva, Heidelberg, and Sigma, St. Louis, MO. HPLC reagents were purchased from Fluka, Buchs; dithioerythritol was from Roth, Karlsruhe; chemicals for dansylation and SDS–polyacrylamide gel electrophoresis were from Serva, Heidelberg, and guanidine hydrochloride (ultrapure) was from Schwarz/Mann, Orangeburg, NY. Procion Green H-E4BD was a gift of ICI, Organic Division, London. Buffer substances and all other reagents were of A-grade purity, obtained from Merck, Darmstadt. Bidistilled water was used throughout. Buffer solutions were degassed and saturized with nitrogen.

Enzyme Concentration and Activity. Enzyme concentrations of native lactate dehydrogenase were determined spectrophotometrically on the basis of $A^{0.1\%}_{280nm} = 1.40$ (Jaenicke & Knof, 1968). Absorption coefficients of the proteolytic products were deduced from the absorbance change at 595

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; F 14, F 21, and F 34, fragments of LDH with M_r 13.5K, 21.5K, and 33.5K, respectively; Gdn·HCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; LDH-M_4, lactate dehydrogenase (isoenzyme from porcine skeletal muscle); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; TL, thermolysin; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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nm upon binding of Serva Blue G, with the native enzyme as standard (Bradford, 1976). The latter method was also applied to determine the concentrations of individual fragment samples.

Enzyme activity was monitored in 0.1 M sodium phosphate, pH 7.6 (standard buffer), plus 0.84 mM pyruvate and 0.2 mM NADH at 366 nm in a thermostated recording Eppendorf spectrophotometer at 25 °C. Activity measurements of the proteolytic dimer in the presence of stabilizing salt made use of 2 M ammonium sulfate. Thermolysin activity was measured in 0.1 M Tris·HCl buffer, pH 8.0 (35 °C), and in 0.1 M sodium phosphate buffer, pH 7.6, plus 1 mM dithioerythritol (10 °C), with casein as substrate (Matsubara, 1970).

Methods. Dissociation of lactate dehydrogenase (initial concentration 4.5 mg/mL) was performed by short-term incubation at acid pH (5-fold dilution in 1 M glycine/H_3PO_4, pH 2.3, plus 1 mM dithioerythritol, 4 min at 20 °C, followed by 1-min incubation at 10 °C). Reconstitution was initiated by 40-fold dilution of the dissociated enzyme with standard buffer (final pH 7.6) at 10 °C. Limited proteolysis during reassociation was achieved by adding freshly prepared aqueous solutions of thermolysin (1 mg/mL) to the reconstituting enzyme [for details, see Girg et al. (1981, 1983a)].

To optimize the homogeneity of the proteolytic dimers, varying lactate dehydrogenase to thermolysin molar ratios (>1:10) and incubation times (≤30 min) were applied. Molar concentrations of LDH-M_4 refer to the subunit M_r of 35K. Proteolysis was stopped by adding 5% (v/v) of the total volume of a 0.21 M EDTA solution in 0.1 M sodium phosphate, pH 7.6 (final EDTA concentration 10 mM). The cleavage of the reconstituting subunits was monitored by time-dependent activity measurements in the absence and in the presence of 2 M $(NH_4)_2SO_4$ (Girg et al., 1983a). The yield of proteolytic dimers was estimated by assuming 40% and 70% specific activity for the proteolytic dimer and native lactate dehydrogenase in the presence of 2 M $(NH_4)_2SO_4$, respectively (see below).

Separation of proteolytic dimers from reconstituted tetramers, inactivated thermolysin, and smaller fragments was performed by affinity chromatography as described by Girg et al. (1983a), except for the following modifications: Bound dimers and tetramers were eluted with 0.6 and 2.0 M NaCl, respectively. To concentrate the dimer fraction, ultrafiltration (Amicon Miniflo with PM-10 membranes) with subsequent dialysis against water plus 10 mM β-mercaptoethanol at 4 °C and lyophilization were applied. SDS–polyacrylamide gel electrophoresis with 12–18% polyacrylamide gradients was used to characterize the final product. Separation of the constituent parts of the proteolytic dimers, e.g., smaller fragments (originating from nicked dimers), and the 34K truncated fraction was achieved by gel chromatography under denaturing conditions: 0.1 M sodium phosphate, pH 7.6, plus 3 M Gdn·HCl and 100 mM β-mercaptoethanol; Sephadex G-100 superfine, 2.8 × 68 cm, 12 mL/h flow rate. Fractions were collected, dialyzed against 5% (v/v) acetic acid, lyophilized, and stored at −18 °C.

End group analyses with dansylchloride and carboxypeptidase Y, as well as manual Edman degradation, made use of common techniques (Hartley, 1970; Gray, 1972a,b; Hayashi, 1978). Amino acid compositions were determined on a Dionex amino acid analyzer (Model D-500) after acid hydrolysis for 24 h, with norleucine as internal standard. The N-terminal sequences were obtained from automated sequence analyses on a Beckman sequencer (Model 890 M) in the presence of polybrene by the method of Edman and Begg (1967). Phenylthiohydantoin derivatives of amino acids were identified by reverse-phase HPLC with a Waters Model 510 system. For details, see Ericsson et al. (1977) and Hunkapiller et al. (1983).

Denaturation of the proteolytically modified enzyme was accomplished by incubation at low pH, elevated temperature, and high urea or high Gdn·HCl concentrations. Reactivation/reassociation after guanidine denaturation was started by dilution with standard buffer; kinetics were analyzed by taking aliquots at defined times.

Intrinsic protein fluorescence was recorded in a Hitachi Perkin-Elmer MPF 44 A fluorescence spectrophotometer, absorption spectra were recorded in a Cary 118 spectrophotometer, and circular dichroism spectra were recorded in a JASCO 500 A CD spectrograph, equipped with a JASCO DP 500 N data processor and temperature control.

Ultracentrifugal analysis (Beckman Spinco E, equipped with a high-intensity light source and a 10-in. recorder) made use of sedimentation velocity and sedimentation equilibrium experiments, applying double-sector and band-forming cells in An-D and An-G rotors.

Results

Reconstitution of porcine muscle lactate dehydrogenase after acid dissociation has been shown to be determined by a rate-limiting association reaction: The dimer is formed in a fast (diffusion-controlled) process followed by a concentration-dependent association to the native tetramer (Hermann et al., 1981, 1983). Previous data (Girg et al., 1981) have shown that thermolysin digestion of LDH-M_4 during reconstitution leads to reassociation arrest at the dimer stage. End group analysis and SDS–polyacrylamide gel electrophoresis proved the product to be heterogeneous (Grandits, 1979): Subunits were cleaved not only at their N- or C-terminus but also in the interior of the polypeptide chain (Girg et al., 1981).

Preparation of Homogeneous Fragments. In order to achieve homogeneity of the fragments, the conditions of proteolysis were optimized with respect to the thermolysin:lactate dehydrogenase ratio and incubation time. Both variables are essential in fulfilling the requirements for proteolytic reconstitution studies: (i) stability of the folded (native) enzyme toward proteolysis and (ii) fast proteolytic attack of intermediates. The third requirement, instantaneous inactivation of the protease after the proteolytic pulse, is met by the addition of EDTA to a final concentration of 10 mM.

Fast proteolysis of the intermediates of association, as determined by the inhibition of lactate dehydrogenase reactivation, is found to require a minimum thermolysin:lactate dehydrogenase molar ratio of 1:4 at 10 °C. With increasing time of proteolysis, the initially formed truncated chains, F 34, are further cleaved into two smaller fragments, F 21 and F 14 (Figure 1). The fragments become more homogeneous with increasing time of thermolysin digestion, as indicated by the decreased degeneracy of N-terminal residues (cf. Table I). Equal amounts of the three fragments and optimum homogeneity were obtained by adding the protease after 1-min reconstitution with a subsequent thermolysin digestion of 15 min. Under this condition, the final yields of tetramers, F 34, F 21, and F 14 were <6%, 34%, 32%, and 28%, respectively. After isolation of the proteolytic dimers by affinity chromatography on Procion Green, the constituent fragments were separated by gel filtration under denaturing conditions, e.g., in the presence of 3 M Gdn·HCl, as illustrated in Figure 2. The homogeneity of the fragments (as determined by SDS–polyacrylamide gel electrophoresis and densitometry) was >96%.

Table I: Characterization of Proteolytic Fragments of Porcine Muscle Lactate Dehydrogenase[a]

	F 34	F 21	F 14
electrophoretic purity (%)	98.5[b]	100	>96[c]
M_r			
SDS–PAGE	33.5K ± 1K	21K ± 1K	13.5K ± 1K
amino acid analysis	33.5K	21.4K	
N-termini			
5 min[d]	L, K, V, I	L, K, V, I	L, K, V, S
10 min	L, K	L, K	L, V
≥15 min	L	L	L, V
manual Edman degradation	H_2N-LL	H_2N-LLK	H_2N-LL H_2N-LS H_2N-VS
N-terminal sequence[e]	^{11}LLKEEHVPHNKI (70) ^{12}LKEEHVPHNKIT (30)	^{11}LLKEEHVPH (≤100)	(a) ^{11}LLKEEHVPHNKI (60) (b) ^{39}LMKELADEIALVD (10) (c) ^{208}VSLKNLHPELGT (30)
C-terminal residues	F, T, D, L, V	S, D, G, V	
$A_{280nm}^{0.1\%}$ (cm^2 mg^{-1})	1.44 ± 0.2[f]	1.36 ± 0.2[f]	1.47 ± 0.2[f]

[a] Unless otherwise stated a 15-min thermolysin pulse was applied during reconstitution. [b] Sequence analysis (N-terminus) gives evidence for two closely related polypeptide chains. [c] A shoulder in electrophoretic densitograms indicates some heterogeneity; the N-terminal sequence proves F 14 to consist of at least three fragments. [d] Time of proteolysis with thermolysin; increasing incubation time leads to a marked decrease in heterogeneity. [e] Residue numbers according to Eventoff et al. (1977); in parentheses are relative amounts of subfragments (%). [f] In 6 M Gdn·HCl.

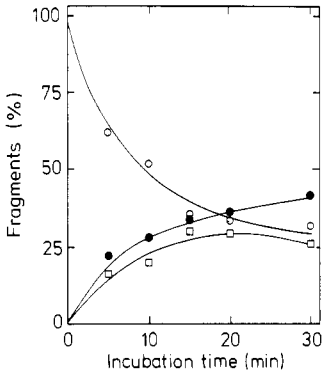

FIGURE 1: Effect of time of incubation with thermolysin during reactivation on yield of different LDH fragments. Proteolysis was initiated by adding thermolysin 1 min after starting reactivation (10 °C). In order to quench the protease, 10 mM EDTA was added at the times given. LDH:TL ratio is 4:1; c_{LDH} = 20 μg/mL. Amounts of fragments (%) are documented for (○) F 34, (●) F 21, and (□) F 14, respectively.

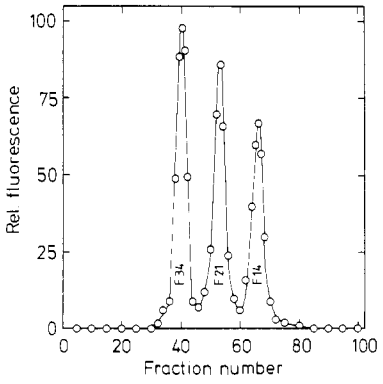

FIGURE 2: Purification of fragments of porcine muscle lactate dehydrogenase after limited proteolysis with thermolysin. Gel chromatography of proteolytic dimers under denaturing conditions (sample buffer was 6 M Gdn·HCl plus 100 mM β-mercaptoethanol in 0.1 M sodium phosphate, pH 7.6; column volume 480 mL; flow rate 12 mL/h; fraction size 4 mL; 20 °C). Elution with 0.1 M sodium phosphate, pH 7.6, plus 3 M Gdn·HCl and 100 mM β-mercaptoethanol.

Characterization of Fragments. Characteristic data for the proteolytic fragments of lactate dehydrogenase are summarized in Table I. There is clear evidence from the molecular mass and the terminal residues that the three fragments belong (i) to the complete polypeptide chain of the subunit lacking a short stretch of both the N- and C-terminal end or (ii) to the two domains upstream and downstream of the "wing region" (Holbrook et al., 1975). The amino acid composition (Table II) corroborates this idea at least for the truncated fragments F 34 and F 21. The N-terminal sequences of the first 9–12 residues are included in Table I.

Table II: Amino Acid Composition of N-Terminal Fragments of Lactate Dehydrogenase after Thermolysin Cleavage

	F 34[a]				F 21[b]	
		calcd for				calcd for
	obsd	^{11}L–^{306}T	^{11}L–^{321}T	^{11}L–^{331}F	obsd	^{11}L–^{196}S
K	25.9	22	24	25	12.0	12
H	10.0	10	11	11	6.1	6
R	12.9	10	10	10	10.6	9
B	28.5	30	31	31	18.9	19
T	10.3	9	11	11	5.0	5
S	22.0	22	23	23	17.0	13
Z	28.3	21	25	27	17.2	14
P	13.0	11	12	12	6.7	7
G	27.7	24	24	25	15.5	16
A	16.6	17	19	19	9.8	10
C		5	5	5		4
V	32.7	36	36	36	16.7	20
M	8.0	8	8	8	5.3	6
I	19.6	22	22	23	10.9	14
L	33.1	30	32	34	18.2	20
Y	7.0	7	7	7	4.0	4
F	6.4	6	6	7	4.9	5
W		5	5	6		2

[a] As taken from end group analysis with carboxypeptidase Y, there is no unique C-terminus. Assuming the three components ^{11}L–^{306}T, ^{11}L–^{321}T, and ^{11}L–^{331}F to be present in equal amounts, the average molecular weight of F 34 is 34027. [b] The "wing" starts at position ~194; ^{197}V is the first thermolysin-sensitive peptide bond. The observed C-terminal residues (S, D, G, V) fit with either ^{197}V, ^{199}V, or ^{203}V. The respective molecular weights are 20472, 20668, and 20901.

As a consequence of the neighboring leucine residues in the "N-terminal arm", F 34 yields a ragged sequence starting from ^{12}Leu, apart from the main component with ^{11}Leu as N-terminus. F 14 turns out to be a mixture of three fragments: fragments a and b originate from the N-terminal (coenzyme binding) domain, while fragment c represents a C-terminal fragment starting from ^{208}Val (see Table I). Considering the amino acid composition together with the fragment size and the C-terminal residues, we may conclude that fragment F 34 comprises residues 11/12 to 321/331, fragment F 21 comprises residues 11/12 to 200 ± 3, and fragment F 14 (c) comprises residues 208 to ~331.

Table III: Comparison of Native Porcine Muscle Lactate Dehydrogenase with the F 34 Dimer in 0.1 M Sodium Phosphate, pH 7.6, 20 °C

	native LDH-M_4	F 34 dimer
M_r		
sedimentation equilibrium	140K ± 4K[a]	65K ± 3K
SDS–polyacrylamide gel electrophoresis	35K	34K
$A^{0.1\%}_{280nm}$ (cm^2 mg^{-1})	1.40 ± 0.05[a]	1.40 ± 0.2
specific activity (IU/mg)		
standard assay	480	<2
in 2 M $(NH_4)_2SO_4$	400	160 ± 10
K_M(pyruvate) (μM)	62[b]	174[b]
K_M(NADH) (μM)	67[b]	135[b]
θ_{222nm} (×10^{-3} deg cm^2 dmol^{-1})	−12.0	−11.5

[a] In 0.2 M sodium phosphate, pH 7.0 [cf. Jaenicke and Knof (1968)]. [b] In the presence of 2 M $(NH_4)_2SO_4$ and 0.2 mM NADH or 0.84 mM pyruvate, respectively; 25 °C.

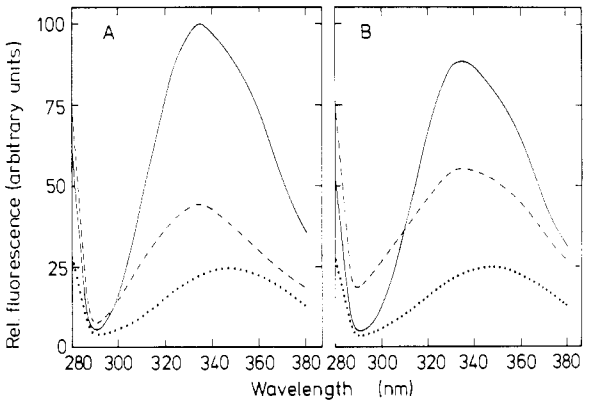

FIGURE 3: Fluorescence emission spectra of porcine muscle lactate dehydrogenase (LDH-M_4) and the F 34 dimer in their native, denatured and reconstituted states. Native conditions: 0.1 M sodium phosphate, pH 7.6, and 10 mM β-mercaptoethanol. Denaturing conditions: 6 M Gdn·HCl plus 100 mM β-mercaptoethanol in 0.1 M sodium phosphate, pH 7.6. Enzyme concentration 7.6 μg/mL; 20 °C; λ_{exc} = 280 nm. (—) Native LDH; (– –) reconstituted F 34 dimer; (···) denatured LDH and F 34 fragment. (A) In the absence of $(NH_4)_2SO_4$; (B) in the presence of 1 M $(NH_4)_2SO_4$.

After solubilization of lyophilized "F 34 fragments" by 6 M Gdn·HCl (0.1 M sodium phosphate, pH 7.6, plus 100 mM β-mercaptoethanol), "F 34 dimers" can be reassociated by 1:100 dilution in 0.1 M sodium phosphate, pH 7.6, plus 10 mM β-mercaptoethanol. In Table III a comparison of the native enzyme and the F 34 dimer is given.

The circular dichroism of the F 34 dimer at 215–250 nm closely resembles the far-UV spectrum of the native enzyme, with essentially the same mean residue rotation at 222 nm (Table III). Obviously, the F 34 dimer represents a structured entity with most of the polypeptide backbone in its native conformation. Considering the high level of residual structure preserved upon acid denaturation (Zettlmeissl et al., 1979, 1981), this result is not unexpected.

As shown in Figure 3, the fluorescence emission of the F 34 dimer in the absence of additives is lowered as compared to the native enzyme; the emission maximum is unchanged (λ_{max} = 336 nm). Addition of stabilizing salt [1 M $(NH_4)_2SO_4$] diminishes the spectral difference: the fluorescence emission of the native enzyme is decreased while that of F 34 shows a significant enhancement; this is in accordance with the decrease in tetramer activity and the appearance of dimer activity previously reported for the enzyme and its proteolytic fragments at high concentrations of structure-making ions (Girg et al., 1983a). In the presence of 6 M guanidine, the spectra of both native lactate dehydrogenase and its F 34 fragment coincide; λ_{max} is shifted to 348 nm.

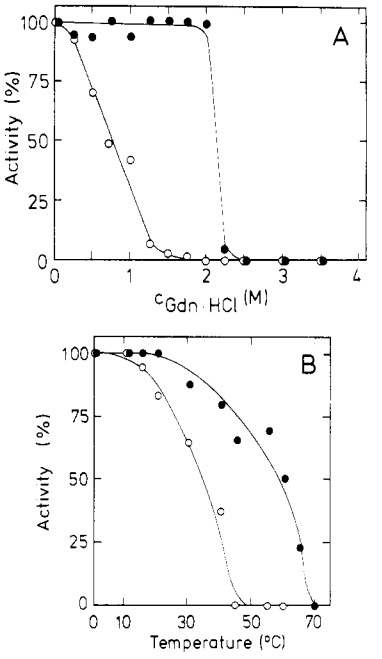

FIGURE 4: Deactivation of porcine muscle lactate dehydrogenase (LDH-M_4) and the F 34 dimer by Gdn·HCl and temperature. Incubation time: 1 h under conditions given. To compare the native and the truncated enzymes, activity was measured in the presence of 2 M $(NH_4)_2SO_4$. (●) LDH-M_4; (○) F 34 dimer. (A) Guanidine denaturation in 0.1 M sodium phosphate, pH 7.6, plus 1 M $(NH_4)_2SO_4$ and 100 mM β-mercaptoethanol; enzyme concentration 0.8 μg/mL; 20 °C. (B) Heat denaturation in 0.1 M sodium phosphate, pH 7.6, plus 1 M $(NH_4)_2SO_4$ and 10 mM β-mercaptoethanol; enzyme concentration 1.2 μg/mL.

The observation that structure-making ions induce enzymatic activity of the F 34 dimer (Table III) suggests a relatively high degree of structural flexibility of the fragment dimer, as compared to the native tetramer. To investigate the relative stability, deactivation of native lactate dehydrogenase and the F 34 dimer was measured at varying Gdn·HCl concentrations. Stock solutions were incubated in 0–3.5 M Gdn·HCl [0.1 M sodium phosphate, pH 7.6, plus 100 mM β-mercaptoethanol and 1 M $(NH_4)_2SO_4$, 20 °C]. Subsequent activity measurements in the presence of 2 M $(NH_4)_2SO_4$ show that deactivation of the fragment dimers starts at low c_{Gdn} (transition midpoint at c_{Gdn} = 0.8 ± 0.1 M) with very low cooperativity, while the native enzyme exhibits high cooperativity with a transition midpoint at c_{Gdn} = 2.15 ± 0.10 M (Figure 4A). A similar difference in stability is observed in heat-inactivation experiments in the presence of 1 M $(NH_4)_2SO_4$. The respective transition temperatures (50% activity after 1-h incubation) are 35 °C for F 34 dimers and 60 °C for the native enzyme (Figure 4B). The half-times of the corresponding inactivation kinetics at 55 °C are of the order of 1 min and several hours (4 h), respectively.

Reconstitution. As shown by the reconstitution to F 34 dimers, proteolytic fragments of lactate dehydrogenase exhibit an intrinsic tendency to undergo self-assembly and at least partial regain of native properties. On the basis of the specific activity of the fragment dimers in the presence of 2 M $(NH_4)_2SO_4$, solubilization in 6 M Gdn·HCl and subsequent renaturation by dilution (1:100, final c_{Gdn} = 0.06 M) yield up to 40% reactivation. In these experiments, final values were taken after 3.5–15 h.

In order to determine the *kinetics* of reconstitution of F 34, reactivation was measured as a function of temperature and enzyme concentration (Figure 5). At all temperatures, the rate of reactivation does not vary upon varying the protein

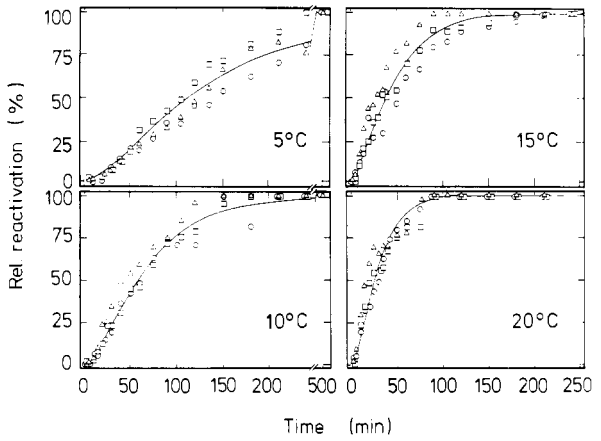

FIGURE 5: Reconstitution of the F 34 fragment of porcine muscle lactate dehydrogenase upon varying protein concentration and temperature. Reactivation after solubilization and denaturation in 6 M Gdn·HCl plus 100 mM β-mercaptoethanol by 1:100 dilution with 0.1 M sodium phosphate, pH 7.6, plus 10 mM β-mercaptoethanol and a residual Gdn·HCl concentration of 0.06 M at 5, 10, 15, and 20 °C. Protein concentration during reactivation was 1.4 (○), 4.5 (□), and 21 (△) μg/mL. Enzyme activity at 25 °C in the presence of 2 M $(NH_4)_2SO_4$. The absolute regain of activity was 30%, 37%, and 15% for c = 1.4, 4.5, and 21.0 μg/mL, respectively.

Table IV: Temperature Dependence of Kinetics of Reconstitution of the Proteolytic Fragment F 34 after Denaturation in 6 M Gdn·HCl[a]

temp (°C)	k_1 (s^{-1})	k_2 (s^{-1})
5	$(6.4 \pm 0.2) \times 10^{-4}$	$(1.40 \pm 0.05) \times 10^{-4}$
10	$(9.6 \pm 0.2) \times 10^{-4}$	$(3.00 \pm 0.06) \times 10^{-4}$
15	$(10.8 \pm 0.4) \times 10^{-4}$	$(5.14 \pm 0.2) \times 10^{-4}$
20	$(12.0 \pm 0.5) \times 10^{-4}$	$(10.0 \pm 0.4) \times 10^{-4}$
activation energy (kcal/mol)	7.2	19.7

[a] Computer fit of the data for consecutive first-order reactions according to eq 1.

concentration by a factor of 15, which proves that the association step cannot be involved as the rate-determining reaction.

Since reconstitution does not proceed beyond the dimer, the minimum kinetic scheme is

$$2\mathcal{M} \xrightarrow{k_1} 2M' \xrightarrow{k_2} 2M \xrightarrow{fast} D \qquad (1)$$

with $\mathcal{M}$ = denatured monomer, M′ = structured monomer without correct subunit interface, M = structured monomer with correct subunit interface, and D = enzymatically active proteolytic dimer. The scheme does not consider the rapid monomer–dimer equilibrium proposed for the reconstitution of intact lactate dehydrogenase (Hermann et al., 1981). Attempts to fit the data for consecutive first-order reactions are illustrated by the full lines in Figure 5; the corresponding kinetic constants are given in Table IV. As previously observed for the reassociation of intact chains, the subsequent monomer association is of the order of a diffusion-controlled reaction (Hermann et al., 1983). Except for the resolution into individual folding steps, the result is in agreement with the kinetic mechanism reported earlier for the intact enzyme (Zettlmeissl et al., 1981; Jaenicke & Rudolph, 1983; Jaenicke, 1984).

In the overall reconstitution of native lactate dehydrogenase, it has been clearly established that the rate-limiting steps are the folding of the monomer (now described by k_1 and k_2) and the dimerization of the dimer to form the native tetramer. The slow folding step of the F 34 fragment, which is characterized by $k_2 = 10 \times 10^{-4}$ s^{-1} at 20 °C, is almost identical with the rate constant of 8×10^{-4} s^{-1} observed for the monomer folding of the intact chain [cf. Zettlmeissl et al. (1982)].

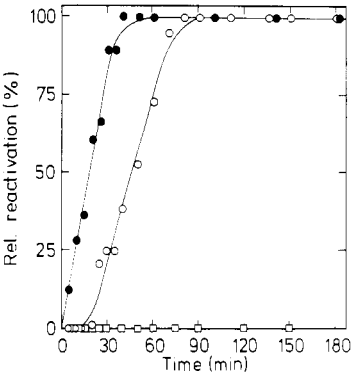

FIGURE 6: Reconstitution of equimolar amounts of the F 14 and F 21 fragments of porcine muscle lactate dehydrogenase after solubilization and denaturation in 6 M Gdn·HCl plus 100 mM β-mercaptoethanol. Reactivation in 0.1 M sodium phosphate, pH 7.6, plus 10 mM β-mercaptoethanol; 20 °C. Enzymatic assay in the presence of 2 M $(NH_4)_2SO_4$. (○/●) Joint reactivation at 10/20 μg/mL total enzyme concentration and 0.06/0.12 M residual Gdn·HCl concentration, respectively. Absolute regain of activity ~6%, corrected for inhomogeneity of F 14. (□) Separated reactivation at 20 μg/mL enzyme concentration and 0.12 M residual Gdn·HCl concentration. After 2-h incubation, the two separate solutions of the fragments were mixed and subsequently tested for enzyme activity.

In previous experiments (Girg et al., 1981) a certain percentage of the proteolytically modified dimers has been shown to be internally nicked, giving rise to smaller fragments, which may be separated under denaturing conditions (SDS–polyacrylamide gel electrophoresis or gel filtration in 3 M Gdn·HCl). In this study, folding and association of the separate domains were investigated by (i) an asynchronous and (ii) a synchronous approach.

(i) Renaturation of the separate fragments, F 21 and F 14, and subsequent mixing of the products do not yield any measurable activity. Instead, an increase in light scattering is observed that proves "wrong aggregation" to compete with correct reconstitution [cf. Zettlmeissl et al. (1979)]. The observation is confirmed by high-speed sedimentation equilibrium experiments.

(ii) Joint reconstitution of the N- and C-terminal fragments yields enzymatic activity. Starting from equimolar quantities of the denatured fragments, 1:100 and 1:50 dilution with standard buffer (pH 7.6) yields up to 6% reactivation, correcting for inhomogeneity of the F 14 fragments. As depicted in Figure 6, the kinetics of reactivation are concentration dependent, in contrast to the results obtained for F 34 (Figure 5). Since the nicked polypeptide chain contains the coenzyme binding domain and (part of) the substrate binding domain on two separate fragments, a rate-determining bimolecular step is to be anticipated.

Discussion

The high-resolution crystal structure of lactate dehydrogenase shows that the tetrameric enzyme represents a dimer of two dimers (Holbrook et al., 1975). The two half-molecules are held together by N-terminal "arms", the absence of which causes the closely related mitochondrial malate dehydrogenase to constitute a stable dimer (Rossmann et al., 1975).

Attempts to produce artificial dimers of lactate dehydrogenase by limited proteolysis of the native enzyme failed because of the restricted accessibility of the N-terminal sequence to proteolytic attack (Jeckel et al., 1973). On the other hand, it has been demonstrated that limited proteolysis during

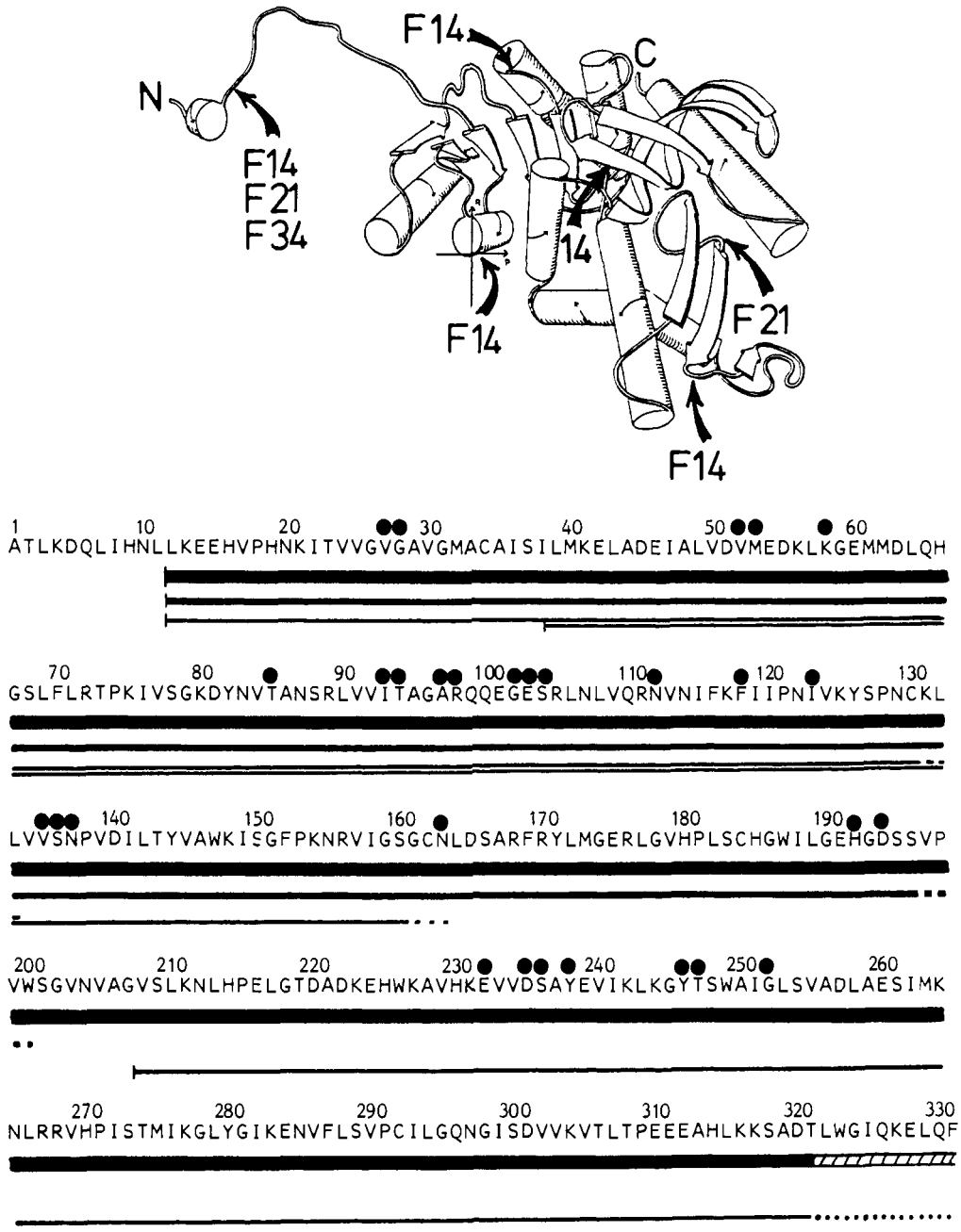

FIGURE 7: Schematic representation of one subunit of dogfish lactate dehydrogenase when viewed along the molecular Q axis (looking from inside the molecule outward). N and C indicate the (acetylated) N-terminus and the C-terminus, respectively. Cleavage points of the three fragments F 34, F 21, and F 14 are marked by arrows. (Below) Amino acid sequence of porcine muscle LDH. Fragments are characterized by (thick bars) F 34, (medium bars) F 21, and (thin bars) F 14. Hatched stretches designate heterogeneous C-terminal ends. Residues involved in ligand binding are characterized by (●) [cf. Holbrook et al. (1975) and Eventoff et al. (1977)].

reconstitution does yield stable dimers, which are enzymatically inactive and no longer able to acquire the native tetrameric state (Girg et al., 1981, 1983a). They are still capable of binding to a dinucleotide affinity column, which suggests that the molecule contains the characteristic Rossmann fold of native dehydrogenases.

Reactivation experiments with native LDH-M_4 show that the unmodified dimeric intermediate on the pathway of reconstitution exhibits high catalytic efficiency in the presence of structure-stabilizing salt (Girg et al., 1983b). In the case of the proteolytic dimers, stabilizing additives like 2 M ammonium sulfate restore the active conformation in a similar way so that ~40% of the activity of the native tetramer is reached (Girg et al., 1983a). Obviously, the proteolytic dimer possesses a native-like structure with anomalously high structural flexibility of its polypeptide backbone. Apart from being less rigid, the proteolytic dimer has been shown to be partially composed of "nicked" chains (Girg et al., 1981). As shown by activity transport measurements in the ultracentrifuge, both the proteolytic dimers and the F 34 dimers sediment as 2S particles, suggesting that cleavage of the polypeptide chain at the level of the dimer does not interfere with the state of association reached on the pathway of reconstitution (Girg et al., 1983a).

Due to the heterogeneity of the proteolytic fragments, no detailed analysis of the distinct cleavage points along the polypeptide chain had been presented previous to this work. In this study a higher degree of homogeneity was achieved after extended proteolysis. End group analysis gave only one single N-terminus for both the F 34 and the N-terminal F 21 fragment. F 14 consists of the C-terminal region (beyond residue 209), apart from two different segments originating

from the N-terminal end of the polypeptide chain. The major components confirm the domain structure of the enzyme (Holbrook et al., 1975; Jaenicke et al., 1981).

By use of the amino acid composition of the individual fragment chains together with the established N-terminal sequences, a satisfactory localization of the cleavage points is provided; with respect to the C-termini, certain ambiguities remain. Figure 7 summarizes the results.

In comparison with the native enzyme, all three fragments as well as the F 34 dimer show decreased stability. For example, sedimentation equilibrium experiments are accompanied by slow aggregation at high concentrations (at the bottom of the cell) so that no true equilibrium can be achieved. Similarly, the far-UV circular dichroism spectra are perturbed by the increase in turbidity. Obviously, the increased flexibility generates aggregation sites that in the native enzyme are either inaccessible or involved in intersubunit contacts stabilizing the "dimer of dimers" structure.

The circular dichroism of the F 34 dimer in its native state provides evidence for a native-like secondary structure of the truncated polypeptide chain (Table III). On the other hand, the tertiary structure seems to be affected by structural flexibility, as indicated by the change in fluorescence emission (Figure 3), as well as the decreased stability toward thermal and guanidine denaturation (Figure 4).

The domains of both the intact and the nicked F 34 fragments represent rigid units that are protected against proteolytic attack even after long incubation with thermolysin. In this respect, the F 34 dimer is found to be closely similar to the native tetramer. Only the unfolded polypeptide chain is cleaved. Even as separate entities, the domains (F 14 and F 21) are stable units capable of joint reconstitution. Obviously, their tertiary structure is sufficiently well-defined to provide recognition sites for their proper assembly.

With respect to the reconstitution of unmodified lactate dehydrogenase, previous experiments have shown that the monomer $\rightarrow$ tetramer transition may be quantitatively described by a consecutive unibimolecular kinetic scheme, corresponding to rate-determining folding and association reactions (Jaenicke & Rudolph, 1983; Jaenicke, 1984). Under certain conditions, the dimer may be accumulated as a stable entity (Jaenicke et al., 1981; Girg et al., 1981). As indicated by a variety of approaches, monomer association is close to diffusion controlled, thus proving the dimerization of the dimer, i.e., the formation of the native tetramer to be the rate-determining association reaction. A slow folding step has been clearly attributed to the formation of "structured monomers" capable of subunit recognition (Jaenicke, 1983). The present experiments confirm these findings for the well-characterized fragment F 34 and for the mixture of F 21 and F 14. As shown by ultracentrifugation (Table I), the final product of reconstitution is the 65K dimer. Its formation is found to be independent of the concentration of the F 34 fragment, in accordance with rate-determining folding steps at the monomer level and subsequent (diffusion-controlled) dimerization (Figure 5).

Attempts to fit the kinetic data with one single first-order rate constant failed. Therefore, a sequence of two consecutive isomerization reactions according to eq 1 were assumed. As shown in Figure 5, the calculated profile fits the experimental data. The activation energies estimated from the temperature dependence in the range from 5 to 20 °C are 7.2 and 19.7 kcal/mol for k_1 and k_2, respectively.

The kinetic constants (Table IV) are in agreement with previous folding data (Hermann et al., 1981; Zettlmeissl et al., 1982; Jaenicke & Rudolph, 1983). The yield of reconstitution was slightly below the value observed for the unmodified tetramer and may be explained by the decreased stability of the F 34 dimer and its increased tendency to form aggregates.

Comparing the reconstitution of the truncated and the nicked proteolytic dimer, a number of similarities are observed. Both fragments show partial activity in the presence of structure-stabilizing salt; both bind to a dehydrogenase-specific affinity column, suggesting that the active center of the native enzyme is present at the level of the dimer, independent of the full integrity of its polypeptide chain.

The fact that the smaller fragments (F 21 and F 14) retain the potential of generating the correct tertiary structure corroborates the idea that proteins with sufficiently large polypeptide chains "fold by parts", with subsequent "merging of domains" as a consecutive reaction. Clearly, the domains contain sufficient structural information, even without the connecting stretches of the chain, to establish the specific intermolecular contacts required to stabilize the native three-dimensional structure. Similar results have been reported for β-galactosidase, lysozyme, penicillinase, tryptophan synthase, and other enzymes (Goldberg, 1969; Arnon & Sela, 1969; Taylor & Silver, 1976; Teale & Benjamin, 1977; Robson & Pain, 1976a,b).

Apart from the previously mentioned similarities, one significant characteristic of the nicked dimer is worth mentioning: in contrast to the intact native polypeptide chain and the F 34 fragment, joint reconstitution of F 21 and F 14 is found to be concentration dependent. Considering the distribution of the ligand binding sites on F 21 and F 14 (Figure 7), one would expect that the formation of the active center of the enzyme requires both fragments to merge. The observed kinetic mechanism is in agreement with this hypothesis: as shown by the concentration-dependent sigmoidal profiles, bimolecular complementation is rate determining, in addition to isomerization reactions at the monomer level.

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Equilibrium and Kinetic Measurements of the Conformational Transition of Reduced Thioredoxin†

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ABSTRACT: The single disulfide bond in *Escherichia coli* thioredoxin was reduced by reaction with a 20-fold excess of reduced dithiothreitol at neutral pH and 25 °C. For some measurements, reduced thioredoxin was further reacted with iodoacetamide to alkylate the cysteinyl residues. The denaturation transitions of oxidized, reduced, and reduced alkylated thioredoxin were observed by using far-ultraviolet circular dichroic and exclusion chromatographic measurements. Cleavage of the disulfide bond lowers the stability of the native thioredoxin to denaturation by about 2.4 kcal/mol, and subsequent alkylation lowers the stability by a further 1.6 kcal/mol. The kinetics of the conformational change of reduced thioredoxin in guanidine hydrochloride were observed by using exclusion chromatography at moderate pressure and 2 °C. Analyses of single and multimixing protocols are consistent with a predominant nonnative configuration in the denatured state and the transient accumulation of a compact nativelike intermediate during refolding. The intermediate can incorporate the nonnative configuration and can accommodate its isomerization. No compelling chromatographic evidence was found for a conformation having an elution time different from that characteristic for either the native or the denatured protein.

The crystallographic model of *Escherichia coli* oxidized thioredoxin (Holmgren et al., 1975) indicates that the single disulfide bond in the protein bridges the first and fourth residues of a type III reverse turn involving residues 32–35. Such a reverse turn likely persists in the denatured protein and could in principal direct the folding of the polypeptide into its native conformation. In this report, we describe equilibrium and kinetic measurements of the conformational transition of *E. coli* thioredoxin having its single disulfide bond reduced. We find that the conformational features of soluble native

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