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Folding and stability of different oligomeric states of thiamin diphosphate dependent homomeric pyruvate decarboxylase

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Abstract

The folding and stability of recombinant homomeric (α-only) pyruvate decarboxylase from yeast was investigated. Different oligomeric states (tetramers, dimers and monomers) of the enzyme occur under defined conditions. The enzymatic activity is used as a sensitive probe for structural differences between the active and inactive form (mis-assembled forms, aggregates) of the folded protein. Unfolding kinetics starting from the native protein comprise both the dissociation of the oligomers into monomers and their subsequent denaturation, which could be monitored by stopped-flow kinetics. In the course of unfolding, the tetramers do not directly dissociate into monomers, but via a stable dimeric state. Starting from the unfolded state, a reactivation of homomeric pyruvate decarboxylase requires both refolding to monomers and their correct association to enzymatically active dimers or tetramers. The reactivation yield under the in vitro conditions used follows an optimum behavior.

Keywords: Homomeric recombinant pyruvate decarboxylase; Oligomeric states; Association/dissociation; Protein folding; Thermodynamics; Kinetics

1. Introduction

Pyruvate decarboxylase (EC 4.1.1.1) is an enzyme within the glycolytic pathway in fermenting cells. Thiamin diphosphate as coenzyme and magnesium ions are essential for its enzymatic function [1,2]. The differently associated states of the yeast enzyme have been investigated by X-ray small angle scattering [3,4]. The native enzyme from yeast is a heterotetramer with a molecular mass of 240,000 Da [5,6]. This tetramer consists of a dimer of dimers [7]. A pH dependence has been found for both the oligomerization (association/dissociation equilibrium) and the co-factor binding ability [4,8].

The crystal structures of pyruvate decarboxylase from yeast and that of other ThDP (thiamin diphosphate) dependent enzymes have been published in recent years [7,9–13]. These enzymes have similar folding to that of the ThDP binding

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pocket, involving hydrogen bonds to the pyrimidine and thiazolium ring and coordination of the magnesium ion at the diphosphate moiety [13]. In pyruvate decarboxylase, the co-factor ThDP is non-covalently bound in the 'V' conformation [14,15] at the interface between two monomers involving their respective α and γ domains [7]. Therefore, residues from these two subunits interact with the co-factor and are required for its binding.

The quaternary structure of PDC (pyruvate decarboxylase) from yeast and other organisms has been studied by synchrotron solution X-ray scattering (SAX) [3]. Different oligomeric states of the homomeric recombinant enzyme could be populated using a denaturant and have been characterized according to their structural and catalytic properties [16]. Addition of the co-factors ThDP and magnesium ions shifts the association/dissociation equilibrium to the tetrameric state, indicating a stabilizing effect of the co-factors. A treatment of denaturant (urea) on homomeric pyruvate decarboxylase revealed a transition profile comprising two well-separated transitions both for the holoenzyme and the apoenzyme [17]. Using a protein concentration of 70 μg/ml (1.2 μM), dimers occur at 0.5 M urea and monomers occur at 2.0 M urea. The first transition could be related to the population of folded monomers, the second transition to their unfolding [16].

In this work, the folding and assembly of the recombinantly produced homomeric enzyme, the protein of *PDC1* (gene coding for pyruvate decarboxylase from *Saccharomyces cerevisiae*) is studied using both functional (enzymatic activity) and optical methods in monitoring structural changes. The approach is related to folding studies of oligomeric proteins as a succession of uni- and bimolecular reactions [18–20]. A thermal denaturation of pyruvate decarboxylase is accompanied by an enzymatic inactivation and was measured by circular dichroism and the decrease in enzymatic activity. Furthermore, the thermodynamics (stability) of the monomers were measured by equilibrium unfolding using urea. The differently associated states of pyruvate decarboxylase, which can be populated at distinct denaturant concentrations, are covered transiently in folding kinetics. Previously, it could be shown that the dimer is the smallest catalytically active unit of PDC [16]. A reactivation of pyruvate decarboxylase starting from the unfolded state comprises the refolding of monomers and their association to dimers and tetramers, respectively. The reactivation was studied in terms of dependence on different conditions and additives.

2. Experimental

2.1. Preparation and purification of homomeric pyruvate decarboxylase

The production of the enzyme and its purification was performed as described by Killenberg-Jabs et al. [17,21] with slight modifications. The clear supernatant after the rupture, ammonium sulfate precipitation and dialysis was subjected to ionic exchange chromatography on Fractogel® EMD TMAE-650 S (Merck) equilibrated with 20 mM Bis-Tris (bis-[2-Hydroxyethyl]liminotris [hydroxymethyl]-methane) buffer, pH 6.0, 1 mM DTE (dithioerythritol), 0.1 mM ThDP. The protein was eluted by a discontinuous ammonium sulfate gradient. Active fractions were collected, concentrated and underwent gel filtration on Superdex™ 200 (Pharmacia), previously equilibrated with 50 mM sodium dihydrogen phosphate buffer, pH 6.0. Active fractions were concentrated to final concentrations above 5 mg/ml on Filtron 10 kDa, aliquoted and stored at −80 °C. The final yield was approximately 58% (average from 15 preparations) with a specific activity of 74 U/mg. In the course of the preparation, pyruvate decarboxylase was checked by SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and activity determination. All concentrations of the enzyme were related to the molecular mass of the monomer (60,000 Da). The preparation of the apoenzyme was carried out according to the method of Killenberg-Jabs and co-workers [21,22].

2.2. Optical methods

Absorbance spectra of the protein and activity determinations were carried out on an UV/Vis

(ultra violet/visible) spectrophotometer DU 70 (Beckman) or 941 (Kontron).

The intrinsic fluorescence was recorded from 295 to 450 nm on excitation at 280 nm at different denaturant concentrations using a F-4500 Hitachi fluorimeter at 20 °C. The bandpass for both the excitation and the emission monochromators was set at 5 nm, the scan speed at 240 nm/min, and the response time automatically adapted by the device. All fluorescence spectra were corrected according to the supplier's recommendation using Rhodamine B as a standard. The relative fluorescence intensity at 320 nm was used for monitoring urea unfolding.

Light scattering as a probe for aggregation was performed by setting both the excitation and emission monochromators of the fluorimeter at 360 nm using a bandpass of 2 nm for both monochromators.

Circular dichroism spectra of the protein were recorded on a Jasco J710, Jasco J720 and an Aviv spectropolarimeter using cuvettes of 0.1 cm (far UV, near UV) and 1 cm (near UV) optical pathlength. Spectra were acquired at a scan speed of 20 or 50 nm/min, a slit width of 1 or 2 nm and a response time of 1 or 4 s. The ellipticity at 222 nm was used for monitoring urea unfolding and thermal denaturation. Temperature denaturation curves of the protein were carried out in 20 mM sodium dihydrogen phosphate buffer, at the respective pH, using thermostated cuvettes of 0.1 cm (far UV) optical pathlength and a temperature gradient of 50 °C/h. The holoenzyme at pH 8.0, incubated with 200 mM $MgSO_4$, was studied in 20 mM Tris/HCl buffer.

2.3. Activity measurements

The enzymatic activity was determined by a coupled optical test using ADH (alcohol dehydrogenase from yeast) and NADH (reduced nicotinamide–adenine dinucleotide) [23]. In order to study the influence of different conditions and additives on the reactivation, the enzyme was at first denatured at 8 M urea or 6 M GdmCl (guanidinium chloride) in 50 mM PIPES (piperazine-*N*,*N*′-bis-[2-ethanesulfonic acid]) buffer, pH 6.6. Refolding and reassociation were started by 1:100 dilution in renaturation buffer (100 mM PIPES buffer, 1 mM DTE) at the respective pH, ionic strength, additives and protein concentration to be checked. Aliquots of the reactivation mixture were taken at different times. The recovery of the enzymatic activity corresponded directly to the reactivation process. The yield of reactivation was related to the initial activity of the protein before denaturation. The co-factor dependence of reactivation was performed in 50 mM PIPES buffer, pH 6.6, containing 1 mM DTE at a total protein concentration of 10 μg/ml at 20 °C.

2.4. Equilibrium unfolding by urea and temperature denaturation

Urea unfolding curves of the monomers of homomeric pyruvate decarboxylase were fitted to a two-state model, in which the native (N) and denatured (D) states are present, with the program Kaleidagraph™ and a Macintosh computer. The values for $[D]_{50\%}$ (concentration of denaturant, where 50% of the protein is unfolded) and $\Delta G^{H_2O}_{D-N}$ (free energy of unfolding in the absence of denaturant) were determined by the linear extrapolation method:

$$\Delta G_{D-N} = \Delta G^{H_2O}_{D-N} - m \cdot [D] \quad (1)$$

$$\Delta G_{D-N} = -RT \cdot \ln K_{D/N} \quad (2)$$

$$K_{D/N} = \frac{(X_N - X)}{(X - X_D)} \quad (3)$$

where $[D]$ is the concentration of denaturant, ΔG_{D-N} is the free energy of unfolding at a given denaturant concentration, m is the slope of the plot in the transition region, X_N is the value of the specified optical signal (fluorescence at 320 nm or ellipticity at 222 nm) of the folded protein, X_D is that of the denatured protein and X is that of the protein at a given denaturant concentration. $K_{D/N}$ represents the equilibrium ratio of unfolded/native protein species at any specified denaturant concentration, T is the absolute temperature and R is the gas constant. Evaluation of equilibrium constants in the transition region requires an extrapolation of the pre- and post-unfolding baselines into the transition region. Considering the intercepts (n_N,

n_D) and the slopes (m_N, m_D) of the pre- and post-unfolding regimes, the following equation was used for fitting urea denaturation curves [24–28]:

$$X = \frac{(n_N + M_N \cdot [D]) + (N_D + M_D \cdot [D]) \cdot \exp\left(\frac{-m \cdot ([D]_{50\%} - [D])}{RT}\right)}{1 + \exp\left(\frac{-m \cdot ([D]_{50\%} - [D])}{RT}\right)} \quad (4)$$

Temperature denaturation curves were evaluated by the derivatives using the program Kaleidagraph™ and a Macintosh computer giving the respective thermal transition midpoints.

2.5. Stopped-flow kinetics

Kinetic experiments for folding studies were performed on an Applied Photophysics Bio-Sequential DX.18 MV stopped-flow spectrometer (Leatherhead, UK). The change in the total fluorescence above 305 nm (cut-off filter) was recorded upon excitation at 280 nm and at 20 °C. The photomultiplier was set at 550 V. Experiments were carried out in 50 mM PIPES buffer, pH 6.6, 1 mM DTE, 200 mM $MgSO_4$ and a final protein concentration of approximately 1.4 μM (1+10 dilution of 15.4 μM stock concentration).

Protein folding occurs as a multi-step transition and can be described as:

$$y(t) = \sum y_i \cdot \exp(-k_i t) + y_\infty \quad (5)$$

where $y(t)$ and y_∞ are amplitudes of the optical probe at time t and at equilibrium, respectively, while y_i is the amplitude at zero time and k_i is the first-order rate constant of phase i. Data were fitted to a double exponential first-order reaction. Single jump unfolding experiments were performed starting from both 0 M (unfolding of the dimer/tetramer) and 3.5 M (unfolding of the monomer) urea in unfolding buffer containing different amounts of denaturant. On the other hand, single jump refolding experiments were performed by an 11-fold dilution of 7 M urea-unfolded protein in refolding buffer containing different amounts of urea. At least 4–7 traces were used for averaging, both in unfolding and refolding experiments. The observed rate constants (k_{obs}) of the respective phases are presented in a chevron plot (Fig. 6). The influence of the integral fluorescence of the enzyme on the signal amplitude was checked using different cutoff filters (305 nm, 320 nm, 350 nm). A single wavelength detection was carried out by replacing the second monochromator for the cutoff filter.

3. Results and discussion

3.1. Equilibrium denaturation measurements

The urea equilibrium denaturation of the homomeric pyruvate decarboxylase using a protein concentration of 70 μg/ml (1.2 μM) shows well separated transitions monitored by intrinsic fluorescence, far UV circular dichroism and assayed by the determination of the enzymatic activity [16]. A small change of the optical signal is monitored between 0 and 0.5 M urea, a transition between 0.5 and 2 M urea and a further transition between 2 and 6 M urea. The transition patterns of all spectroscopic probes are similar for both the holoenzyme and the apoenzyme (Fig. 1a,b). The fluorescence maximum remains unchanged at 332 nm up to 0.5 M urea. However, there is an increase in the fluorescence intensity of the intrinsic fluorophores going from 0.5 M urea to 2 M urea, accompanied by a red shift of the maximum to 342 nm. The far UV circular dichroism spectrum of the protein, however, slightly decreases until 2 M urea, reflecting a loss in secondary structure (Fig. 1a,b). From 2 to 6 M urea, the fluorescence intensity decreases and the maximum shifts to 350 nm. The far UV circular dichroism spectrum also approaches a random coil with further decreases in the mean residual ellipticity [16]. There is almost no difference in the secondary structure between the holoenzyme and apoenzyme of homomeric PDC monitored by far UV circular dichroism [22] and some difference in the tertiary structure monitored by fluorescence.

Considering the results from analytical ultracentrifugation, the small change of the transition profile between 0 and 0.5 M urea is attributed to the dissociation of the tetramers to the dimers: the first transition to that of the dimers to monomers

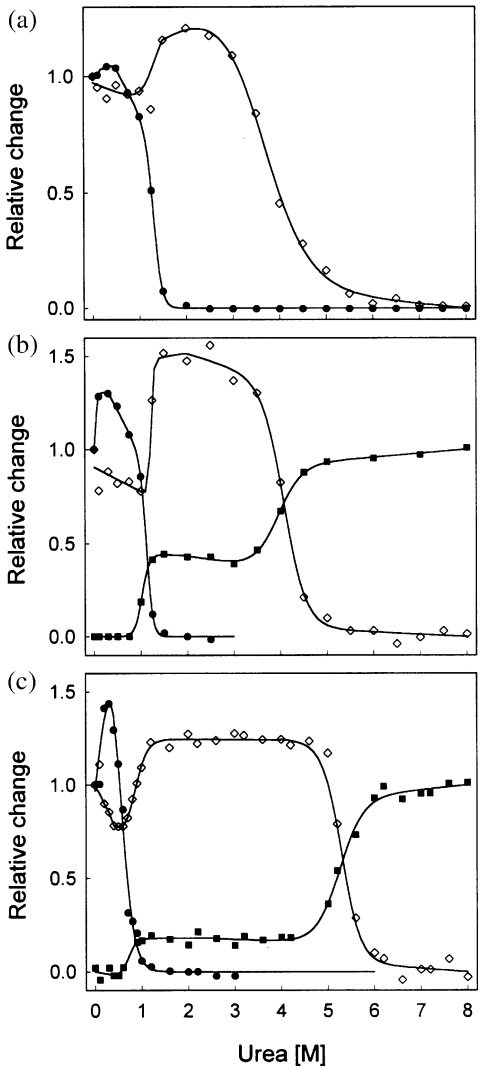

Fig. 1. Equilibrium unfolding of homomeric pyruvate decarboxylase by urea monitored by intrinsic fluorescence at 320 nm (◇), circular dichroism at 222 nm (■) and the enzymatic activity (●). The samples were measured after 20 h incubation at 20 °C. The protein concentration was 70 μg/ml in all measurements. (a) Holoenzyme in 50 mM sodium dihydrogen phosphate buffer, pH 6.0. (b) Apoenzyme in 50 mM sodium dihydrogen phosphate buffer, pH 6.0. (c) Holoenzyme in 20 mM sodium dihydrogen phosphate buffer, pH 6.0 and 200 mM $MgSO_4$. Activity measurements were performed after reconstitution with ThDP under saturating conditions. Normalization was related to the values at 0 M urea.

and the second transition reveals the unfolding of the monomers [16]. The increase in the intrinsic fluorescence in forming monomers is caused by a dequenching of the aromatic amino acids in the contact region of the subunits and the loss of the co-factor ThDP [29,30].

The addition of 200 mM magnesium sulfate shifts the midpoint of the second transition to higher denaturant concentrations (by approx. 1.5 M), which indicates an increase in stability (Fig. 1c). The free energies of denaturation of the monomers determined under different conditions are summarized in Table 1a. Monomers populated at 2 M urea tend to aggregate at that denaturant concentration. Aggregates can be redissolved at higher denaturant concentrations. On the other hand, monomers are stable at 3.5 M urea, where no aggregation is observed. Summarizing, all oligomeric species of homomeric pyruvate decarboxylase can be populated at distinct urea concentrations. Magnesium ions stabilize the monomer, but do not change the concentration range of urea that favors the formation of aggregates (approx. 2 M urea) (Table 1b). This stabilizing effect cannot be replaced by sodium ions. Magnesium ions are known to be coordinated in the active site at the diphosphate moiety of the coenzyme ThDP bound in the interface between two subunits [7,31]. Consequently, these bivalent metal ions are also able to coordinate to other specific functional groups than in the active site region. Therefore, we consider the monomer as the thermodynamic unit that can unfold and refold cooperatively and rationalize, following the two-state assumption (Table 1a) [20].

Near UV circular dichroism measurements have been performed with the apoenzyme of homomeric PDC, as well as canceling out a possible optical interference of the co-factor ThDP in the holoenzyme [21,22]. Considering the dimer/tetramer equilibrium of the apoenzyme in dependence on the protein concentration, the tetramer formation results in a decrease in the mean residual ellipticity (Fig. 2). At a concentration of 9 mg/ml, mostly tetramers are present. An excess of dimers is present at a lower protein concentration of approximately 0.8 mg/ml. The same ellipticity is also measured for the sample at the same protein

Table 1
Equilibrium unfolding and dissociation of recombinant pyruvate decarboxylase by urea

A
Unfolding by urea

Protein and conditions[a]	Unfolding monitored by fluorescence at 320 nm			Unfolding monitored by circular dichroism at 222 nm		
	$[D]_{50\%}$ (mol·l^{-1})	m ($\partial\Delta G/\partial[D]$) (kcal·l·mol^{-2})	$\Delta G^{H_2O}_{D-N}$ (kcal·mol^{-1})	$[D]_{50\%}$ (mol·l^{-1})	m ($\partial\Delta G/\partial[D]$) (kcal·l·mol^{-2})	$\Delta G^{H_2O}_{D-N}$ (kcal·mol^{-1})
Apoenzyme, pH 6.0	4.1±0.1	2.4±0.4	9.8±1.8	4.1±0.1	2.2±0.4	8.8±1.4
Holoenzyme, pH 6.0	3.8±0.1	1.2±0.1	4.6±0.4	n.d.	n.d.	n.d.
Holoenzyme, pH 6.0, 200 mM $MgSO_4$	5.3±0.1	3.4±0.6	17.8±3.1	5.3±0.1	2.0±0.2	10.8±1.2
Holoenzyme, pH 8.0, 200 mM $MgSO_4$	5.2±0.1	1.5±0.1	7.6±0.6	4.9±0.1	1.4±0.2	7.0±1.0
Holoenzyme, reversibility, pH 6.0, 200mM $MgSO_4$	5.3±0.1	2.9±0.1	15.4±0.3	n.d.	n.d.	n.d.

B
Dissociation induced by urea

Protein and conditions[a]		$[D]_{50\%}$	
	Monitored by activity[b]	Monitored by fluorescence at 320 nm	Monitored by circular dichroism at 222 nm
Apoenzyme, pH 6.0	1.1±0.1	1.2±0.1	1.1±0.1
Holoenzyme, pH 6.0	1.3±0.1	1.4±0.1	n.d.
Holoenzyme, pH 6.0, 200 mM $MgSO_4$	0.6±0.1	0.9±0.1	0.7±0.2
Holoenzyme, pH 8.0, 200 mM $MgSO_4$	0.7±0.1	0.9±0.1	0.9±0.1

[a] All experiments were performed in 50 mM sodium dihydrogen phosphate buffer at 20 °C.
[b] Activity measurements were performed by a coupled optical test using ADH/NADH as described in Section 2.
n.d.: not determined.

concentration and at 0.5 M urea, where dimers are populated. A further increase in the urea concentration above 2 M results in a dissociation of the dimers into monomers. However, this disassembly causes no significant change in the near UV circular dichroism spectrum, as seen in the spectrum recorded at 3.5 M urea (Fig. 2). The near UV circular dichroism of the apoenzyme shows an overall change in the asymmetry of the aromatic chromophores only of the tetramers in comparison to the dimers and monomers. The population of different oligomeric species (dimers and monomers) of homomeric PDC at distinct denaturant concentrations (urea) reflects the denaturant dependence of their corresponding equilibrium constants.

3.2. Thermal denaturation and inactivation

The secondary structure of homomeric pyruvate decarboxylase decreases on heating from 10 to 95 °C monitored by far UV circular dichroism (Fig. 3a). The shape of the spectrum measured at 95 °C, however, does not correspond to a random coil state, but rather to a compact denatured state [32] containing some secondary structure (Fig. 3b). At pH 6.0, the holoenzyme (T_m=327.3 K) and the apoenzyme (T_m=317.3 K) differ in melting by approximately 10 degrees. The T_m of the holoenzyme at a protein concentration of 70 μg/ml (1.2 μM), pH 6.0 and 0.5 M urea, where dimers are populated, is 323.3 K and approaches that of the apoenzyme. The apoenzyme at a protein concentration of 70 μg/ml (1.2 μM) and pH 6.0 melts by only 2 degrees higher than at pH 8.0 (T_m= 315.3 K). At pH 8.0, the co-factor ThDP does not longer bind to the enzyme. This explains why T_m of the holoenzyme is similar to the transition temperature of the apoenzyme at the same pH value (T_m=313.8 K). The presence of 200 mM magnesium sulfate has only little effect on the

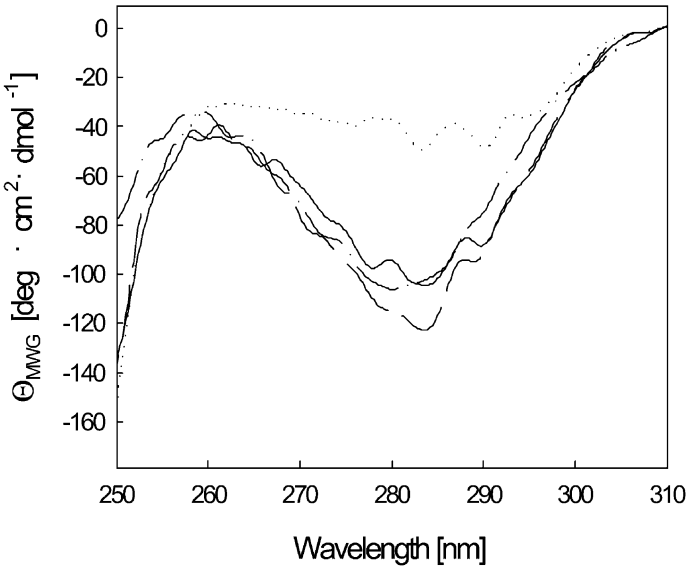

Fig. 2. Near UV circular dichroism spectra at different concentrations of homomeric pyruvate decarboxylase: 9 mg/ml (·····); 0.8 mg/ml (—); and 0.8 mg/ml (– – -) in the presence of 0.5 M urea and 0.8 mg/ml in the presence of 3.5 M urea and 200 mM $MgSO_4$ (– ·–). All measurements were performed in 20 mM sodium dihydrogen phosphate buffer, pH 6.0. The optical pathlength was 0.1 cm at a protein concentration of 9 mg/ml and 1 cm at 0.8 mg/ml.

thermal stability of the holoenzyme, both at pH 6.0 (T_m=328.6 K) and pH 8.0 (T_m=316.3 K). No significant stabilizing effect of the bivalent metal ion could be detected in contrast to the denaturant unfolding. The protein does not aggregate in the course of the thermal denaturation, but after moderate cooling to the start temperature. The two-state assumption cannot be considered for data investigation because of the irreversibility of the thermal denaturation. The single transition comprises both the global and the local thermal denaturation process of homomeric pyruvate decarboxylase.

The kinetics of the thermal inactivation of homomeric pyruvate decarboxylase, however, probe the global thermal denaturation only, and are recorded by the loss of the catalytically active structure, which is strongly influenced by additives and follow first-order kinetics (Table 2). The inactivation at high initial protein concentrations of 1 mg/ml (16.7 μM) is monophasic (Fig. 4). The stabilizing effect of magnesium ions at a concentration of 0.1 M cannot be replaced by sodium ions at the same concentration and might be related to structure stabilization by their interaction with acidic functional groups of the protein. Furthermore, the rate constant of inactivation of the apoenzyme at pH 6.0 is the same as determined for the holoenzyme treated at pH 8.0. Consequently, binding of the co-factor ThDP results in a more stable protein conformation of the homomeric pyruvate decarboxylase [17].

3.3. Unfolding and refolding kinetics

We studied the folding of homomeric pyruvate decarboxylase using stopped-flow kinetics. The progress curves of the unfolding reaction show a fast and a slow folding phase and can be fitted to double exponential first-order kinetics. In starting the unfolding reaction from the associated state at 0 M urea, a protein concentration dependence of the observed rate constants is measured, because the association state of the enzyme is concentration dependent at the startpoint. After a first fast increase, a slower decrease is monitored for the unfolding reaction by a single wavelength detection of the fluorescence at 320 nm (Fig. 5a). The

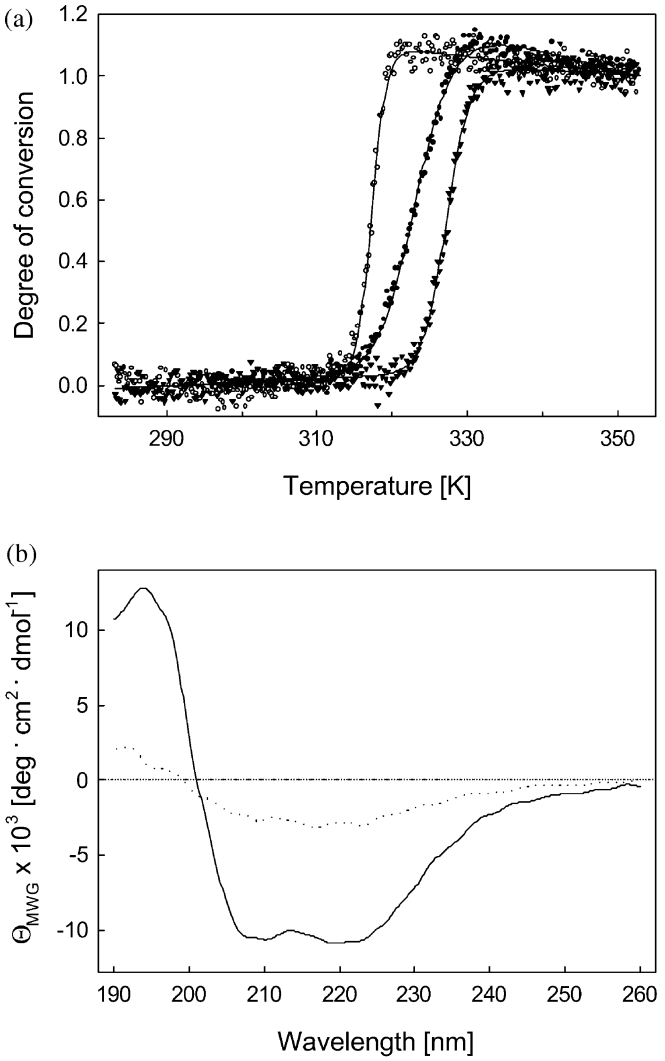

Fig. 3. Temperature denaturation curves of homomeric pyruvate decarboxylase monitored by far UV circular dichroism. (a) Transition profiles of holoenzyme (▼), apoenzyme (○) and holoenzyme in the presence of 0.5 M urea (●). (b) Far UV circular dichroism spectra of the enzyme at 10 °C (—) and 95 °C (·····). All measurements were performed in 50 mM sodium dihydrogen phosphate buffer, pH 6.0. The optical pathlength was 0.1 cm at a protein concentration of 70 μg/ml.

first increase in fluorescence represents the fast formation of the monomers, and the subsequent decrease their slower unfolding. These oligomeric states, which can be populated by equilibrium unfolding, also occur transiently in kinetics, proceeding as a fast disassembly (loss of activity) and a subsequent unfolding process (loss of secondary and tertiary structure).

In contrast, no protein concentration dependence of the kinetics (within the PDC concentration range used) is observed in starting the unfolding reaction from folded monomers at 3.5 M urea. Using the integral fluorescence as optical probe, however, an overall decrease of the signal is monitored (Fig. 5b) according to the optical properties of the populated monomers [16]. On the

Table 2
Heat inactivation of recombinant pyruvate decarboxylase

pH	Protein	Conditions	k (s^{-1})
6.0	holoenzyme	–	0.00252
6.0	holoenzyme	5 mM ThDP	0.00196
6.0	holoenzyme	200 mM $MgSO_4$	0.00009
6.0	holoenzyme	200 mM Na_2SO_4	0.00119
6.0	holoenzyme	5 mM ThDP, 0.5 M urea	0.00508
7.0	holoenzyme	5 mM ThDP	0.0176
7.0	holoenzyme	5 mM ThDP, 200 mM $MgSO_4$	0.000002
8.0	holoenzyme	5 mM ThDP	0.0378
6.0	apoenzyme	–	0.0321

The protein (1 mg/ml) was incubated in 20 mM sodium dihydrogen phosphate buffer at 50 °C. Activity measurements of aliquots taken from the incubation mix were performed by the coupled optical test using ADH/NADH as described in Section 2 at 30 °C. The experimental curves were fitted to a single exponential first-order reaction.

other hand, their refolding was measured in starting from the denaturant-unfolded protein and jumping to lower urea concentrations (up to 3.5 M). The observed rate constants derived from both the unfolding and refolding measurements of the monomers are denaturant dependent and give a chevron with a turning point at approximately 5.3 M urea

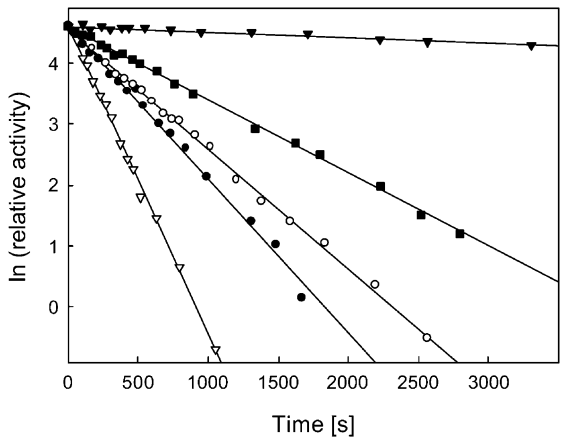

Fig. 4. Influence of ThDP and different metal ions on the thermal inactivation of homomeric pyruvate decarboxylase in 20 mM sodium dihydrogen phosphate buffer, pH 6.0 at 50 °C. The protein concentration was 1 mg/ml, without additives (●), in the presence of 0.5 M urea and 5 mM ThDP (▽), 5 mM ThDP (○), 0.1 M Na_2SO_4 (■) and 0.1 M $MgSO_4$ (▼). The loss in enzymatic activity was determined by the coupled optical test, as described in Section 2.

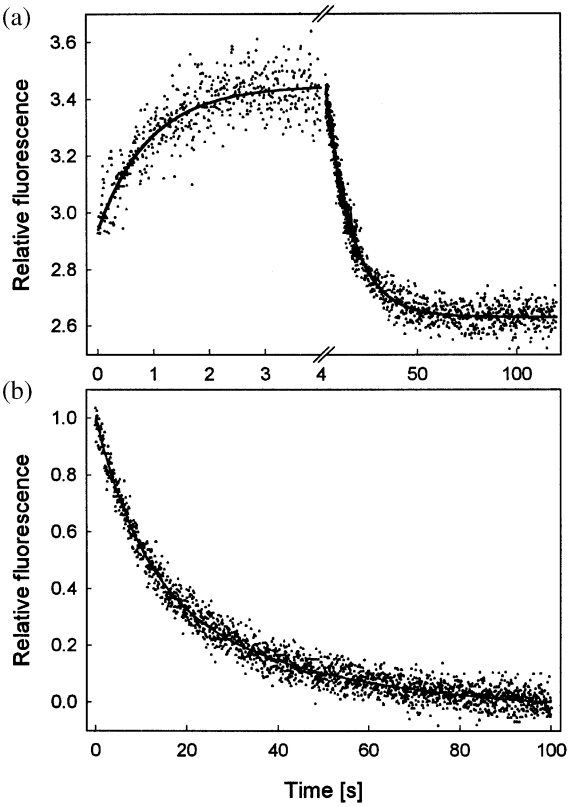

Fig. 5. Unfolding kinetics of homomeric pyruvate decarboxylase. (a) Denaturation in 7 M urea monitored by fluorescence on single wavelength detection. The excitation monochromator was set at 280 nm, the emission monochromator at 320 nm on a stopped-flow machine, as described in Section 2. The final protein concentration was 5 μM in 50 mM PIPES buffer, pH 6.6, 200 mM $MgSO_4$ at 20 °C. (b) Denaturation in 7 M urea starting from 3.5 M urea treated protein monitored by integral fluorescence detection using a cut off filter ($\lambda > 320$ nm) on excitation at 280 nm, as described in Section 2. The final protein concentration was 1.3 μM in 50 mM PIPES buffer, pH 6.6, 200 mM $MgSO_4$ at 20 °C.

(Fig. 6), which corresponds to the transition midpoint of the second transition in the equilibrium unfolding curve (unfolding of the monomers, Fig. 1).

3.4. Reactivation measurements

In multi-subunit proteins, the reactivation consists of both the correct refolding and the reassociation (assembly) as a sum of monomolecular

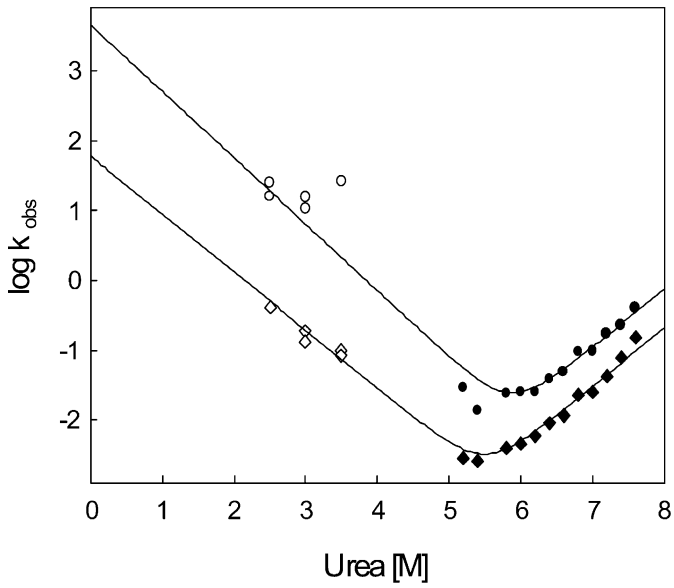

Fig. 6. Chevron of the unfolding and refolding kinetics of homomeric pyruvate decarboxylase. Unfolding kinetics were started from 3.5 M urea treated protein to higher final urea concentrations, refolding kinetics from 7 M urea unfolded protein to lower final urea concentrations on a stopped-flow machine by integral fluorescence detection using a cut off filter ($\lambda > 320$ nm) on excitation at 280 nm, as described in Section 2. The final protein concentration was 1.3 μM in 50 mM PIPES buffer, pH 6.6, 200 mM $MgSO_4$ at 20 °C. Progress curves were fitted to double exponential first-order kinetics, fast phase in unfolding (●) and in refolding (○), slow phase in unfolding (◆) and in refolding (◇).

and bimolecular processes [20]. Hence, the reactivation of denatured homomeric pyruvate decarboxylase comprises the formation of monomers and their correct assembly to catalytically active dimers at least. It follows an optimum behavior with regard to the protein concentration (Fig. 7a), co-factors (Fig. 7b), temperature (Fig. 7c), pH (Fig. 7d) and ionic strength (Fig. 7e). The reactivation yield is approximately 65% under the optimal conditions studied, indicating misfolding and/or aggregation as competition reactions. The optimum protein concentrations for the reactivation of homomeric PDC were between 5 μg/ml (0.08 μM) and 20 μg/ml (0.3 μM) (Fig. 7a). At higher initial concentrations of the protein, the reactivation yield decreases, because of a higher probability in forming aggregates. At the protein concentrations mentioned above, the yield of the reactivation does not change dependent on time, but is lower at concentrations less than 1 μg/ml (0.016 μM). A higher ionic strength prevents aggregation, probably by stabilizing the hydrophobic interactions which are important in the dimer formation. The optimum pH of the reactivation is approximately pH 7.0.

The formation of enzymatically active PDC requires ThDP and magnesium ions, but the reassociation of the monomers to dimers is also possible in the absence of the cofactors. The kinetics of the reactivation have a strong dependence on the protein concentration (Fig. 8a). The bigger the initial concentration of the denatured enzyme, the faster the reactivation. The concentration of ThDP does not influence the reactivation kinetics (Fig. 8b), but the yield (Fig. 7b). With either ThDP (1 mM) or magnesium ions (1 mM) the yield is only approximately 40%; together in the reactivation sample it increases to approximately 50%. There is no difference in the reactivation yield when starting with holoenzyme or apoenzyme as denatured material. The same tendency holds true for the influence of the ionic strength and the pH. The reactivation could be studied only in a small temperature range. At temperatures above 30 °C,

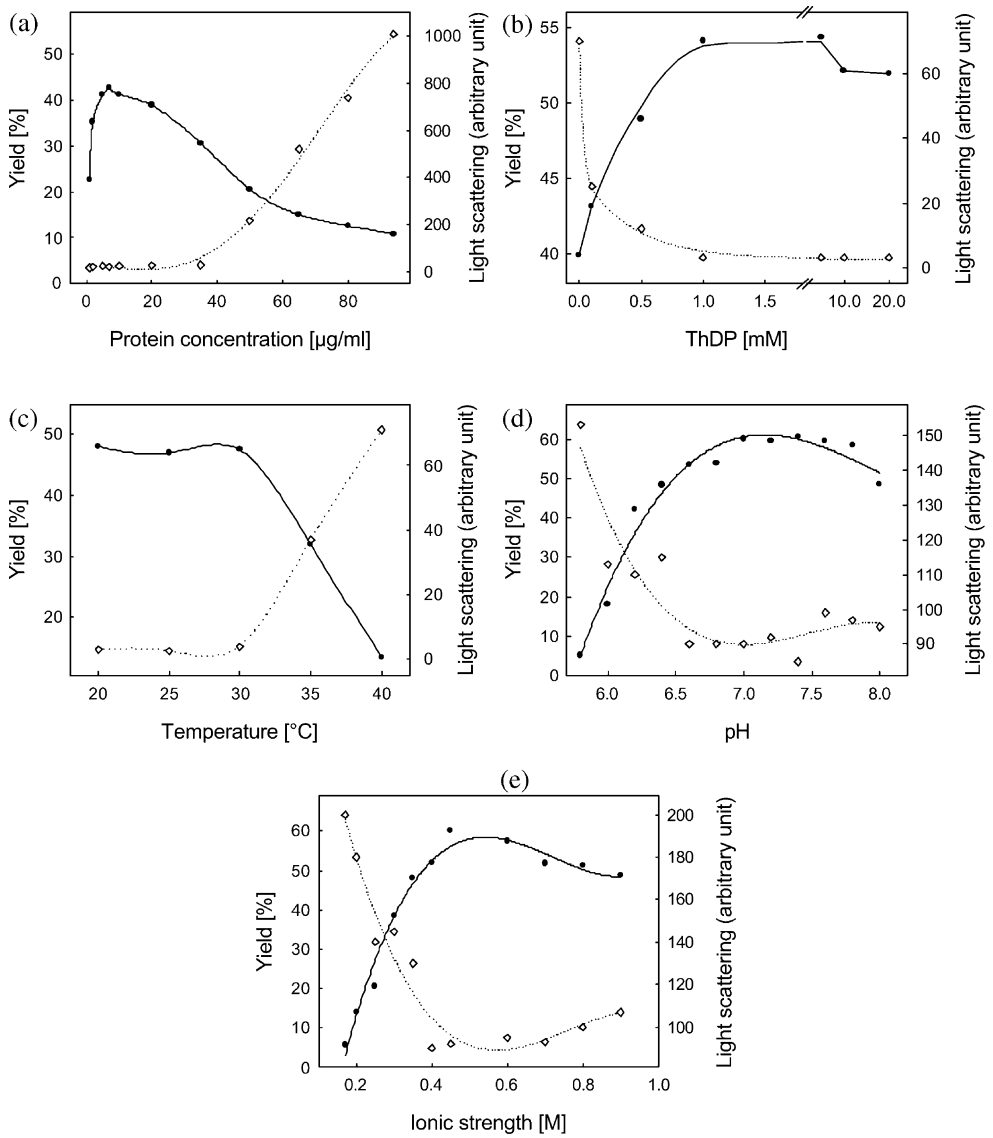

Fig. 7. Reactivation measurements of homomeric pyruvate decarboxylase. Influence of (a) the protein concentration, (b) ThDP concentration, (c) temperature, (d) pH and (e) ionic strength on the reactivation yield (●). Light scattering was determined indicating aggregation (◇). The reactivation was started by 1:100 dilution of 6 M GdmCl denatured protein in renaturation buffer containing 50 mM PIPES buffer, pH 6.6, 1 mM DTE, 1mM EDTA (ethylenediaminetetraacetic acid), 10 mM ThDP, 10 mM $MgSO_4$. Activity measurements were performed by the coupled optical test, as described in Section 2 in 0.1 M PIPES buffer, pH 6.6 containing 10 mM ThDP and 10 mM $MgSO_4$.

aggregation competes with reassociation. In general, the aggregation tendency is directly proportional to the loss in the reactivation yield. At higher temperatures, the reactivation rate is bigger (Fig. 8c), but the yield is lower (Fig. 7c). The co-factor supports the correct assembly of the monomers to dimers and tetramers. Investigating the influence of the co-factor ThDP on the reactivation

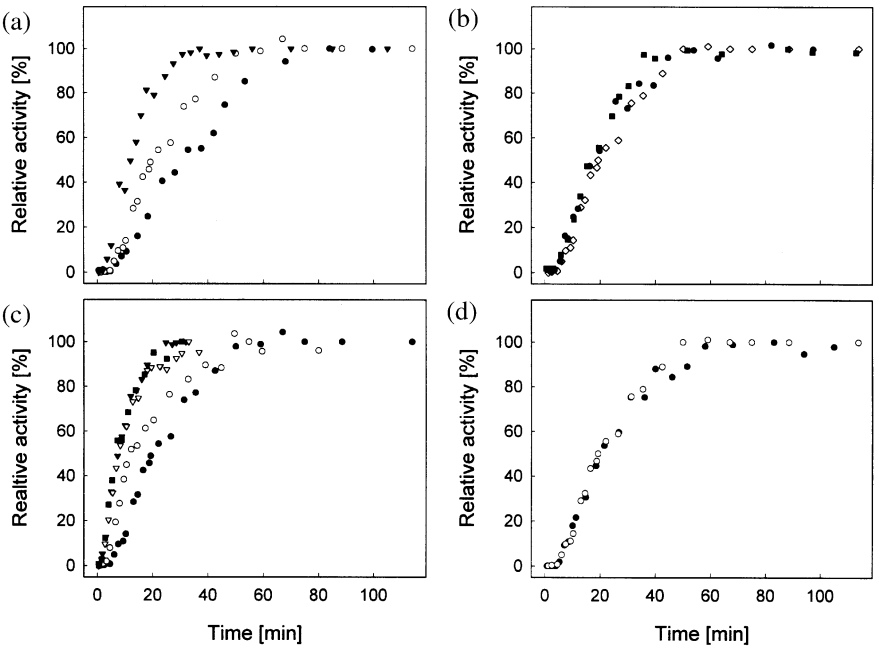

Fig. 8. Reactivation kinetics of homomeric pyruvate decarboxylase. (a) Influence of the protein concentration at 1 μg/ml (●), 5 μg/ml (○) and 10 μg/ml (▼). (b) Influence of the ThDP concentration at 0 M (●), 1 mM (◇) and 5 mM (■). The protein concentration was 5 μg/ml. (c) Influence of the temperature at 20 °C (●), 25 °C (○), 30 °C (▽), 35 °C (▼) and 40 °C (■). The protein concentration was 5 μg/ml. (d) Influence of the pH at pH 6.6 (●) and at pH 7.5 (○). The protein concentration was 5 μg/ml. The reactivation was started by 1:100 dilution of 6 M GdmCl denatured protein in renaturation buffer containing 50 mM PIPES buffer, pH 6.6, 1 mM DTE, 1 mM EDTA, 10 mM ThDP, 10 mM $MgSO_4$. The concentration of ThDP in (b) varied as indicated. Activity measurements were performed by the coupled optical test, as described in Section 2, in 0.1 M PIPES buffer, pH 6.6 containing 10 mM ThDP and 10 mM $MgSO_4$.

kinetics the reaction was started with an apoenzyme. At ThDP concentrations in the optimum range, the profile of the reactivation rate is the same. However, no dependence of the reactivation kinetics of the enzymatically active protein on pH in the ambient range could be observed (Fig. 8d). Furthermore, a lag phase is clearly observed in the profiles of all parameters investigated (Fig. 8), which is related to the concentration-dependent process of the correct assembly of the refolded monomers to enzymatically active dimers and tetramers. The critical step in the reactivation process is very likely the correct association of the monomers under the in vitro conditions used. A guided process of the assembly of the α- and β-subunits of the heteromeric native pyruvate decarboxylase from yeast is assumed to occur under in vivo conditions after protein biosynthesis, and very likely involving the action of the chaperone machinery of the cell to avoid the formation of mis-assembled proteins (aggregates) as competing reactions.

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