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Trehalose counteracts the dissociation of tetrameric rabbit lactate dehydrogenase induced by acidic pH conditions

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ABSTRACT

The lactate dehydrogenase from rabbit skeletal muscle (rbLDH) is a tetrameric enzyme, known to undergo dissociation when exposed to acidic pH conditions. Moreover, it should be mentioned that this dissociation translates into a pronounced loss of enzyme activity. Notably, among the compounds able to stabilize proteins and enzymes, the disaccharide trehalose represents an outperformer. In particular, trehalose was shown to efficiently counteract quite a number of physical and chemical agents inducing protein denaturation. However, no information is available on the effect, if any, exerted by trehalose against the dissociation of protein oligomers. Accordingly, we thought it of interest to investigate whether this disaccharide is competent in preventing the dissociation of rbLDH induced by acidic pH conditions. Further, we compared the action of trehalose with the effects triggered by maltose and cellobiose. Surprisingly, both these disaccharides enhanced the dissociation of rbLDH, with maltose being responsible for a major effect when compared to cellobiose. On the contrary, trehalose was effective in preventing enzyme dissociation, as revealed by activity assays and by Dynamic Light Scattering (DLS) experiments. Moreover, we detected a significant decrease of both $K_{0.5}$ and V_{max} when the rbLDH activity was tested (at pH 7.5 and 6.5) as a function of pyruvate concentration in the presence of trehalose. Further, we found that trehalose induces a remarkable increase of V_{max} when the enzyme is exposed to pH 5. Overall, our observations suggest that trehalose triggers conformational rearrangements of tetrameric rbLDH mirrored by resistance to dissociation and peculiar catalytic features.

1. Introduction

When exposed to environmental stimuli challenging the maintenance of an appropriate osmotic potential, both prokaryotic and eukaryotic cells are known to trigger the synthesis of osmolytes [1]. Altogether, these compounds represent a chemically heterogeneous group, comprising polyols, amino acids, and substituted amines. Moreover, osmolytes can be sub-grouped in two sets, i.e. compatible and counteracting solutes. In particular, the solutes denoted as compatible are those that even at high concentrations do not hamper cellular metabolism, although at the cost of depressing the activity of enzymes [2]. In addition, compounds known to antagonize the harmful effects exerted by some osmolytes (e.g. urea) are defined as counteracting solutes. Among compatible solutes glycerol represents a well-known example, as it was shown for the photosynthetic green alga *Dunaliella viridis*, the glycerol content of which increases as a function of the external NaCl concentration [2]. Conversely, trimethylamine *N*-oxide (TMAO) represents a counteracting solute, which is known to suppress the effects induced by urea on proteins [3].

Trehalose (α-D-glucopyranolsyl-(1 → 1)-α-D-glucopyranoside) is a compatible solute isolated for the first time from *Claviceps purpurea* specimens and thereafter isolated from representatives [4] of the three domains of life [5]. In particular, trehalose shares with other osmolytes the proficiency to stabilize proteins, both under low-water conditions and in aqueous solution. Remarkably, amorphous dehydrated trehalose features glassy properties up to 107 ± 6 °C, undergoes the transition to a rubbery consistency above this temperature (glass transition temperature, T_g), and crystallizes at 184 ± 6 °C [6]. Notably, the T_g of trehalose is significantly affected by the solvent hosting this disaccharide before its dehydration [6]. Nevertheless, among disaccharides trehalose features the highest glass transition temperature [7–9], and therefore is able to form solid amorphous glassy materials at room temperature. This

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peculiarity has been proposed to be responsible for the stabilization exerted by trehalose on bacteria [10,11], liposomes [12,13], and proteins [14–16] stored under desiccated conditions. However, the high T_g of trehalose does not suffice to explain the stabilization that this disaccharide provides to proteins under low-water conditions. It was indeed shown that invertase embedded in rubbery trehalose withstands incubation at 90 °C for 10 h [17]. Interestingly enough, it was also reported that invertase is rapidly denatured when hosted in crystalline trehalose [17]. The plasmamembrane H^+-ATPase from *Kluyveromyces lactis* is an enzyme highly sensitive to freeze-drying and rehydration: after the enzyme is subjected to this process only 4% of the ATPase activity is actually recovered [18]. However, addition of trehalose to the enzyme solution translates into a high resistance to the freeze-drying procedure. In particular, when a trehalose/enzyme molar ratio equal to $5.8 \bullet 10^3$ was used, fully active *K. lactis* ATPase was recovered after the freeze-drying and rehydration procedure [18]. Besides its utility for the stabilization of proteins embedded in low-water systems, trehalose can be conveniently used to inspect, at room temperature, the conformational transitions associated with the reaction steps catalyzed by an enzyme. This was indeed shown for bacterial reaction centers embedded in trehalose glasses featuring different levels of dehydration [19,20].

As previously mentioned, in addition to the stabilization which is able to exert on proteins embedded in low-water systems, trehalose is competent in stabilizing enzymes in solution. Indeed, trehalose was shown to improve the stability of enzymes exposed in aqueous solutions to denaturing conditions induced by high temperatures, chaotropic agents, and freeze-thaw cycles. Concerning the thermal denaturation of enzymes, quite a number of interesting effects triggered by trehalose were reported. The melting temperature (T_m) of bovine pancreatic RNase A was observed to increase by 4.5, 10, and 13.3 °C in the presence of 0.5, 1, and 2 M trehalose, respectively [21,22]. Further, similar dose-response effects triggered by trehalose were detected for cutinase, the T_m of which increased by 4 °C in the presence of 0.5 M trehalose, whilst the addition of 2 M trehalose to a solution containing chicken lysozyme raised the enzyme T_m by 16.5 °C [22,23]. Remarkably, trehalose was observed to activate the catalytic action of enzymes at high temperatures. Indeed, when assayed at 50 or 55 °C, the activity of bovine pancreatic DNase I and of the *Nco*I restriction endonuclease were found to be significantly enhanced (10- and 2-fold, respectively) by the presence of 0.6 M trehalose in reaction mixtures [24]. In addition to the effects exerted on the thermostability of enzymes, it was shown that trehalose counteracts the denaturation of α-chymotrypsin induced by urea. Rather surprisingly, when solutions containing the protease and urea were supplemented with trehalose to obtain a disaccharide/chaotrope molar ratio equal to 0.5, a complete reversion of the effects exerted by urea alone was detected [25]. When freezing is selected as the strategy to store enzymes, a major problem frequently encountered is represented by the loss of activity occurring when the frozen enzyme solutions are thawed. Using β-galactosidase as a model system, the enzyme was found to lose more than 80% of its catalytic performance when subjected to freezing in phosphate-buffered-saline (PBS) buffer at −20 °C for 72 h. However, if 0.29 M trehalose was added to the β-galactosidase solution prior to its freezing no loss of activity was observed [26].

Lactate dehydrogenases (LDHs) catalyze the interconversion of pyruvate and lactate, with the engagement of β-NADH/β-NAD^+ or $FADH_2$/FAD as the cofactor assisting the enzyme in the redox reaction [27,28]. LDHs are oligomeric enzymes assembled into dimeric or tetrameric forms, and exclusively generating L- or D-lactate. Concerning the L-LDHs of vertebrates, two major isoforms were long ago identified, i.e. the heart (H, LDH-B) and the muscle (M, LDH-A) enzyme [29,30]. The rabbit skeletal muscle lactate dehydrogenase is a tetrameric NADH-dependent enzyme, specific for the generation of L-lactate, and denoted LDH-A. Quite recently, the stabilization by trehalose of rabbit LDH-A was investigated under different conditions. The storage stability of freeze-dried rabbit LDH-A was analyzed as a function of the disaccharide supplemented to the enzyme solution prior to freezing [31]. Interestingly, the stabilization provided by trehalose outperformed those exerted by sucrose, maltose and lactose. In particular, this difference was consistent when the enzyme samples were stored at 40 or 60 °C, under fully dry or slightly wet conditions [31]. Surprisingly enough, trehalose was also found to enhance the immobilization yield of rabbit LDH-A [32]. In addition, the effect of trehalose on rabbit LDH-A kinetics was investigated. Performing activity assays in the absence or in the presence of trehalose and using pyruvate as substrate, it was shown that trehalose lowers the K_m for pyruvate and decreases V_{max} [33].

Remarkably, rabbit LDH-A features concentration- and pH-dependent dissociation of the holoenzyme into dimers and monomers [34]. This dissociation is of importance when considering that the dissociated forms of rabbit LDH-A are essentially inactive [35]. Nevertheless, the effect of trehalose on rabbit LDH-A dissociation has not been investigated. Moreover, the evaluation of the competence of trehalose in preventing the dissociation of oligomeric enzymes was not attempted yet. Therefore, we thought it of interest to evaluate the stabilizing effect, if any, of trehalose against the pH-induced dissociation of rabbit LDH-A. To this aim, we performed activity assays over the 5.0–7.5 pH interval, along with a detailed inspection of the dissociation events of rabbit LDH-A triggered by the exposure of the tetrameric enzyme to acidic conditions.

2. Materials and Methods

2.1. Materials

2.1.1. Reagents

Buffers, enzyme substrates, trehalose, maltose, and cellobiose were purchased from Merck-Millipore (St. Louis, MO, USA).

2.1.2. Rabbit lactate dehydrogenase

Lactate dehydrogenase from rabbit skeletal muscle (LDH-A, enzyme suspension in 3.2 M ammonium sulfate, lots 30829424 and 30829428) was obtained from Roche (Basel, Switzerland). LDH-A was dialyzed against 10 mM Tris-HCl (pH 7.5), concentrated to approximately 10 mg/mL with an Amicon ultrafiltration cell equipped with a YM100 membrane, and loaded onto a Superdex-200 gel filtration column (1.6 × 62 cm) previously equilibrated with 10 mM Tris-HCl (pH 7.5). Fractions (0.9 mL) eluted from the column and containing the tetrameric enzyme were pooled, concentrated, and aliquots of the concentrated enzyme solution were stored at −20 °C until used.

2.2. Methods

2.2.1. Activity assays

The enzyme-catalyzed reduction of pyruvate was assayed by determining the decrease in Absorbance at 340 nm related to the oxidation of β-NADH. The extinction coefficient of β-NADH at 340 nm was considered equal to $6.22 \bullet 10^3\ M^{-1}\ cm^{-1}$ [36]. All the assays were performed in duplicate at 25 °C, using a Cary 300 Bio spectrophotometer and starting reactions by enzyme addition. To analyze enzyme kinetics as a function of pH, a universal buffer (containing MES, MOPS, and Tris, 25 mM each) was used [37]. The enzyme kinetic parameters were determined with the Levenberg-Marquardt algorithm in SigmaPlot 14 (Systat Software, San Josè, CA, USA), using the mean of the two values of initial reaction velocity independently determined at each substrate concentration. In particular, the parameter $K_{0.5}$ refers to the substrate concentration corresponding to half-maximal reaction velocity under conditions of cooperative kinetics. The Protein concentration was assayed according to Bradford [38]. It should be mentioned that the Bradford assay translates into rabbit LDH concentration values 20% higher than those obtained spectrophotometrically (using an extinction coefficient at 280 nm equal to 43690 $M^{-1}cm^{-1}$).

2.2.2. Kinetic model for the reaction catalyzed by LDH-A

The dilution-induced dissociation of rabbit LDH-A was analyzed by performing activity assays at pH 5 as a function of enzyme concentration. To this aim, the time course of β-NADH oxidation coupled to the reduction of pyruvate catalyzed by rabbit LDH-A has been interpreted according to the following model, which takes into account the effect of enzyme dissociation on reaction kinetics:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E + P$$

$$E \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} D \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} A$$

Where S, D and A denote β-NADH, the dissociated forms (dimer and monomer) of LDH-A, and the aggregated states of the enzyme, respectively. Both D and A forms were considered devoid of any catalytic activity. It is also important to note that k_{on} is considered a pseudo-first-order rate constant, i.e. the observed reaction kinetics is supposed independent of the concentration of pyruvate. Finally, the simulations were disentangled by constraining the numerical values of the k_1/k_{-1} and the k_4/k_{-4} ratio as equal to 1 and 10^4, respectively.

The model accordingly obtained was used to simulate the kinetics of a set of reactions catalyzed by rabbit LDH-A in the absence of any disaccharide, or in the presence of maltose or trehalose. The simulations were performed by means of the KinTek Global Kinetic Explorer software (KinTek Corp., Snow Shoe, PA, USA).

2.2.3. Dynamic light scattering

Dynamic light scattering experiments were performed with a Malvern Panalytical (Malvern, UK) Zetasizer Nano ZS system. All the measurements were recorded at 25 °C using solutions previously filtered with 0.2 μm filters. Scattering was evaluated at an angle of 173°. Raw data were analyzed with the Zetasizer software (Malvern Panalyticals), release 7.11, and the main relevant peaks accordingly identified were further inspected using the Fityk program [39]. By this means, the area of each peak was normalized to 1 and then deconvoluted into a set of Gaussian distributions, with each component interpreted as a homogeneous sub-population of the enzyme ensemble.

2.2.4. Mass spectroscopy

Immediately before mass spectrometric analyses, aliquots of tetrameric LDH-A stored at −20 °C in 10 mM Tris-HCl (pH 7.5) were thawed and diluted with a buffer containing ammonium acetate and ammonium bicarbonate (5 mM each, AA-AB buffer). Further, the diluted aliquots were subjected to buffer exchange using a HiTrap desalting column (5 mL, Cytiva, Marlborough MA, USA) previously equilibrated with AA-AB buffer poised at pH 5 or 7. Diluted/buffered LDH solutions were then flow-injected to a syringe pump operated at 10 μL/min into a Q Exactive mass spectrometer (Thermo Fisher Scientific, Waltham MA, USA) equipped with a HESI-II ion source. Ions were produced by ESI, paying attention not to interfere with the oligomerization equilibria, according to the following parameters: positive polarity, 2.3 kV spray voltage, 320 °C capillary temperature, 15 auxiliary gas, 1 sheath gas, S-lens RF 50, and the probe was heated at 60 °C. The mass spectrometer was operated in Full Scan mode, performing a 400–6000 Full MS scan at 140,000 resolution (at m/z 200). The observed multicharge ESI spectra were deconvoluted with the mMass software (release 5.5.0 [40]).

3. Results

3.1. Effect of disaccharides on the activity of rabbit LDH-A exerted under different pH conditions

The activity of rabbit LDH-A is known to feature a maximum at pH 6.5 [41], and to undergo a pronounced decrease at pH ≤ 6 induced by the dissociation of the tetrameric enzyme [34]. To further test these observations, we assayed rabbit LDH-A activity in the presence of 125 μM β-NADH and 0.5 mM pyruvate, using a universal buffer poised at pH values ranging from 5 to 7.5. Under these conditions, and in agreement with previous reports, we detected the maximal enzyme performance at pH 6.5 and a low level of catalytic action at pH 5 (Fig. 1A). In particular, the activity at pH 5 was determined as equal to about 10% of that observed over the 6–7.5 pH interval, suggesting the occurrence of an extensive enzyme dissociation under acidic conditions (Fig. 1A). Therefore, we thought it of interest to evaluate the effectiveness, if any, of different disaccharides in counteracting the pH-induced enzyme dissociation. When reaction mixtures were supplemented with cellobiose at concentrations up to 0.25 M, a moderate decrease of enzyme activity was observed at pH 6 and 6.5, whereas in the presence of 10–150 mM of the disaccharide a slight activation (10–20%) was detected (Fig. 1B). Conversely, cellobiose was found to strongly depress the activity of rabbit LDH at pH 5, with the disaccharide at 40–250 mM inhibiting 80–90% of the enzyme action (Fig. 1B). Surprisingly, an even more pronounced inhibition of rabbit LDH-A was determined in the presence of maltose. In particular, when the enzyme was exposed to pH 7.5 or 6.5 in the presence of 250 mM maltose, its catalytic performance was found to decrease by 20 and 45%, respectively (Fig. 1C). Further, at both pH 6 and 5 very strong inhibition was observed, with the enzyme being very sensitive to the presence of 10–50 mM maltose, and performing very poorly in the presence of 250 mM of the disaccharide (Fig. 1C, cf. Fig. 1B). When rabbit LDH-A was assayed in the presence of trehalose, we determined a peculiar dependence of activity as a function of the disaccharide concentration. Indeed, we did not observe major effects at pH 6, 6.5, and 7.5 when reaction mixtures were supplemented with up to 300 mM trehalose, the presence of which at 900 mM did significantly inhibit the enzyme (Fig. 1D). Even more surprisingly, trehalose was found to induce an increase of enzyme activity at pH 5, with an exponential dependence of reaction velocity on disaccharide concentration (Fig. 2). This observation suggests that trehalose is very efficient in counteracting the dissociation of tetrameric LDH-A induced by acidic pH conditions.

3.2. Trehalose affects the kinetic parameters of rabbit LDH-A

To further investigate the action of trehalose on rabbit LDH-A we performed activity assays to determine the enzyme kinetic parameters, at different pH values (5, 6.5, and 7.5) and in the absence or in the presence of 0.4 or 0.8 M trehalose. In particular, we used assay mixtures containing 3.1 nM tetrameric enzyme (12.4 nM subunits), 125 μM β-NADH, and different concentrations of pyruvate. When the activity was tested at pH 7.5 in the absence of trehalose, the enzyme was found to obey Michaelis-Menten kinetics, featuring K_m and V_{max} respectively equal to 750 ± 60 μM and 2100 ± 100 nM/s (Fig. 3A, Table 1). Notably, the presence of 0.4 M trehalose at pH 7.5 did significantly alter both K_m and V_{max}, whose values were determined as significantly lower when compared to those evaluated in the absence of the disaccharide (Fig. 3B, Table 1). Moreover, increasing the concentration of trehalose from 0.4 to 0.8 M triggered a significant further decrease of K_m and V_{max} (Fig.s 3A and 3 B, Table 1).

In agreement with observations that we recently reported [34], rabbit LDH-A was found to feature sigmoidal kinetics when assayed at pH 6.5 in the absence of trehalose (Fig. 3C). In particular, at this pH we determined values for $K_{0.5}$ and V_{max} respectively equal to 150 ± 10 μM and 1900 ± 100 nM/s, and we estimated a value of 1.3 ± 0.1 for the Hill coefficient (Table 1). It is important to mention here that we have previously shown that the pH-dependent transition from Michaelis-Menten to sigmoidal kinetics of rabbit LDH-A represents the outcome of association/dissociation equilibria of the enzyme tetramer [34]. Therefore, we tested whether trehalose is competent or not in affecting the pH-dependent shift from hyperbolic to sigmoidal kinetics of rabbit

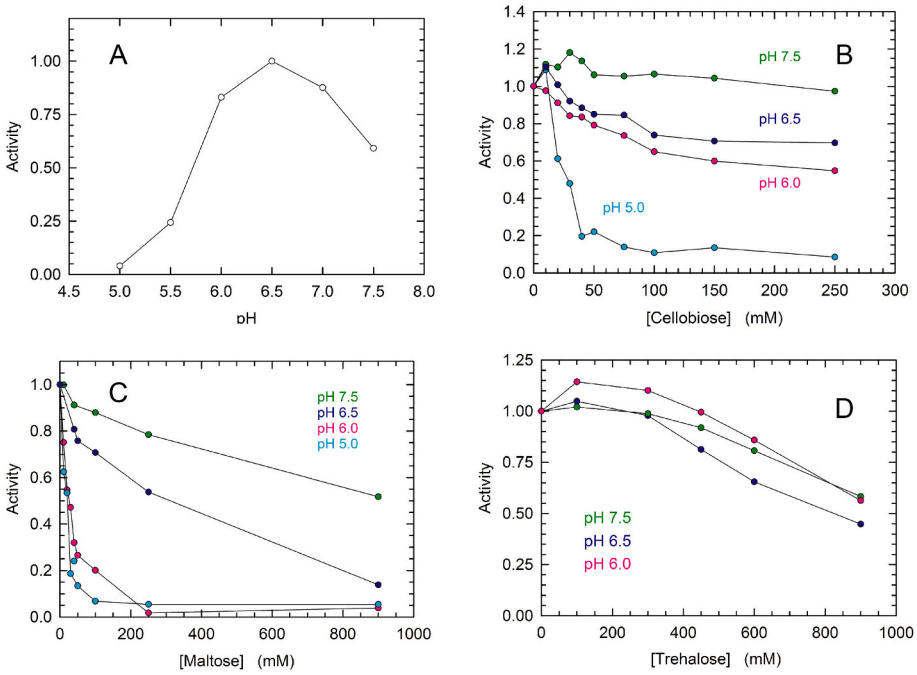


Fig. 1. Effect of different disaccharides on the catalytic activity of rabbit LDH-A.
(**A**) Initial reaction velocities determined as a function of pH in the presence of 125 μM β-NADH, 500 μM pyruvate and 8 nM tetrameric rabbit LDH-A (32 nM subunits). The maximum level of activity was normalized to 1. (**B-D**) Activity exerted by 1.6 nM tetrameric rabbit LDH-A (6.4 nM subunits) determined as a function of pH in the presence of cellobiose (B), maltose (C), or trehalose (D). The extent of activity detected in the absence of cellobiose (B), maltose (C), or trehalose (D) was normalized to 1. Other conditions as for the assays reported in panel A.

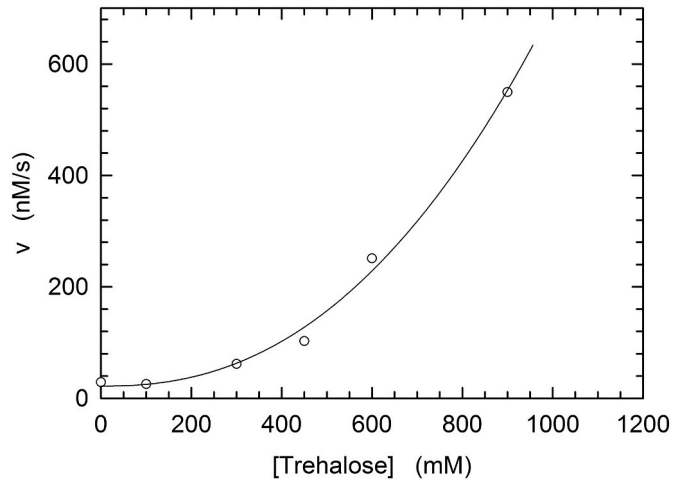


Fig. 2. Effect of trehalose on LDH-A activity exerted at pH 5.
Dependence on trehalose concentration of LDH-A activity assayed at pH 5 in the presence of 125 μM β-NADH, 500 μM pyruvate and 1.6 nM tetrameric rabbit LDH-A (6.4 nM subunits).

LDH-A. When 0.4 or 0.8 M trehalose was added to the reaction mixtures the enzyme was found to obey sigmoidal kinetics (Fig.s 3C and 3D, Table 1). Further, the presence of trehalose, independently of its concentration, triggered a significant decrease of the values of both $K_{0.5}$ and V_{max} (Table 1), with the addition of 0.4 M trehalose being sufficient to elicit a substantial effect on both kinetic parameters (Table 1).

When the activity of rabbit LDH-A was assayed at pH 5 a peculiar dependence on trehalose was observed. Indeed, at this pH value the enzyme took advantage of the addition of 0.4 M trehalose to reaction mixtures, responding with an increase of the V_{max} value from 1400 ± 100 to 3600 ± 200 nM/s, and with a decrease of $K_{0.5}$ from 110 ± 10 to 80 ± 10 μM (Fig.s 3E and 3F, Table 1). However, as also observed at pH 6.5, the presence of 0.4 M trehalose did not induce a transition from sigmoidal to Michaelis-Menten kinetics. Moreover, no significant difference was detected between the values of the Hill coefficient determined for the enzyme action performed in the absence and in the presence of 0.4 M trehalose (Fig.s 3E and 3F, Table 1). When reaction mixtures were supplemented with 0.8 M trehalose quite surprising observations were obtained. First, very high reaction rates (equal to approximately 6 μM/s), were detected at relatively low pyruvate concentrations, i.e. 50-100 μM (Fig. 3F). Also, substrate inhibition occurred in the presence of trehalose at concentrations of pyruvate higher than 300 μM (Fig. 3F). This complex and unexpected behavior was further inspected by performing an additional set of assays, using a different lot of enzyme (see Methods). Remarkably, the dependence of reaction velocity on pyruvate concentration evaluated with the two enzyme lots was found to qualitatively agree (Table 1, Fig. S1).

3.3. Trehalose prevents the dissociation of tetrameric rabbit LDH-A induced by acidic pH conditions

To further inspect the high performance of rabbit LDH-A induced at pH 5 by trehalose, we decided to analyze two potentially interrelated properties of the enzyme: i) the decay of activity as a function of LDH-A residence time at pH 5, in the absence or in the presence of 0.8 M trehalose; ii) the dissociation of the enzyme tetramer induced by acidic pH conditions. To test the decay of enzyme activity, we incubated LDH-A at pH 5 (in MES-MOPS-Tris buffer) in the absence of coenzyme and substrate, and at predetermined time intervals the reaction was started by the simultaneous addition of 125 μM β-NADH and 0.5 mM pyruvate. When this assay was carried out in the absence of trehalose, a rather fast decay of activity was observed. In particular, 80% of the initial activity was lost in 30 s, and a decay rate constant equal to 0.12 ± 0.01 s^{-1} was obtained by fitting a single-exponential equation to the data observed over the first 60 s of incubation (Fig. 4A). This initial fast phase was followed by a further decrease of enzyme activity, the extent of which was determined as equal to ca. 4% after 300 s of incubation at pH 5 (Fig. 4A). The addition of 0.8 M trehalose to the enzyme solution exposed to pH 5 translated into a decay of LDH-A activity much slower when compared to that detected in the absence of the disaccharide. Remarkably, the incubation of the enzyme for 300 s in 0.8 M trehalose at pH 5 triggered a 30% increase of activity over the level initially detected (Fig. 4B). Subsequently to this, further incubation at pH 5 in the presence of trehalose induced a significant decay of the rabbit LDH-A catalytic action (Fig. 4B). However, it should be noted that the rate of this

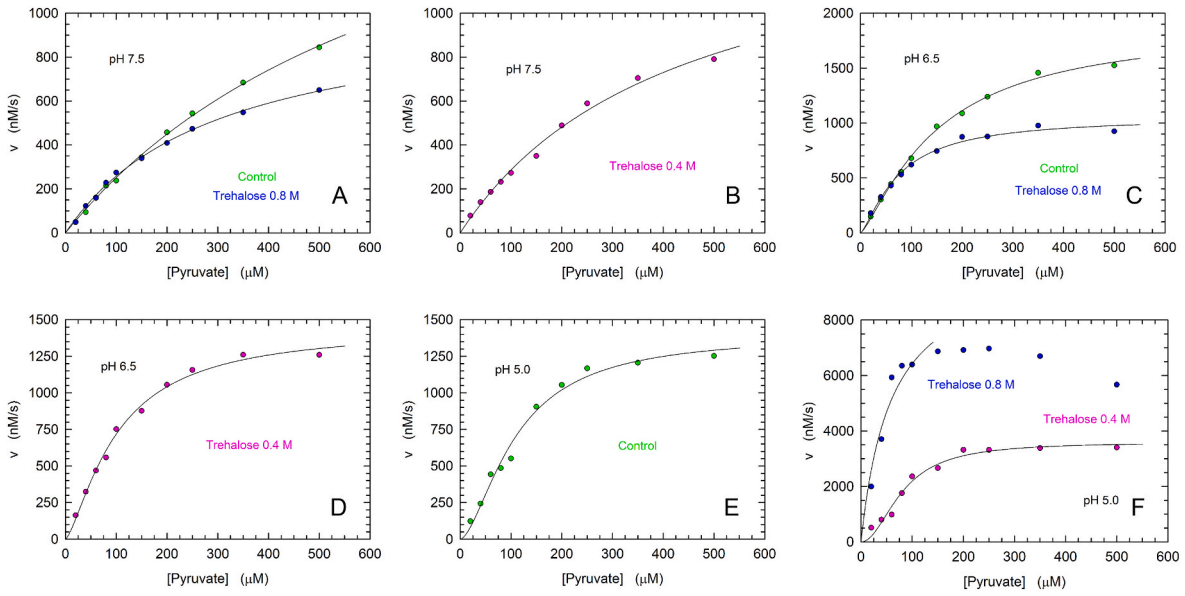


Fig. 3. Kinetics of pyruvate reduction catalyzed by rabbit LDH-A in the absence and in the presence of trehalose.
(**A-F**) The activity assays were performed, at the indicated pH values, in the presence of 125 μM β-NADH, 3.1 nM tetrameric LDH-A (12.4 nM subunits) and variable concentrations of pyruvate. The continuous lines represent the best fits of the Michaelis-Menten or the Hill equation to the experimental observations.

Table 1
Kinetic parameters of rabbit LDH-A.
The Michaelis-Menten or the Hill equation was fitted to the experimental observations shown in Fig. 3. Assays were performed in the presence of 3.1 nM enzyme (tetramer concentration, corresponding to 12.4 nM of subunits). The errors represent the standard deviation associated with the fittings. An independent experiment performed at pH 5 in the presence of 0.8 M trehalose yielded K_m and V_{max} values equal to 70 ± 40 μM and 10000 ± 3000 nM/s, respectively (Fig. S1).

pH	Trehalose (M)	K_m (μM)	V_{max} (nM/s)	Hill
7.5	–	750 ± 60	2100 ± 100	–
7.5	0.4	400 ± 60	1500 ± 100	–
7.5	0.8	300 ± 20	1000 ± 40	–
6.5	–	150 ± 10	1900 ± 100	1.3 ± 0.1
6.5	0.4	100 ± 10	1400 ± 100	1.4 ± 0.1
6.5	0.8	80 ± 10	1100 ± 50	1.4 ± 0.2
5.0	–	110 ± 10	1400 ± 100	1.6 ± 0.2
5.0	0.4	80 ± 10	3600 ± 200	2.0 ± 0.4
5.0	0.8	60 ± 20	10000 ± 2000	–

decay was found to be rather slow, i.e. being appropriately interpreted by a first-order rate constant equal to 0.0020 ± 0.0005 s^{-1} (Fig. 4B). Moreover, it is important to note that after 1 h of incubation at pH 5 the enzyme activity was equal to ca. 35% of the initial level (Fig. 4B).

As previously mentioned, we attempted a straightforward analysis of the dissociation of rabbit LDH-A. In particular, we analyzed by Dynamic Light Scattering (DLS) LDH-A samples exposed to solutions poised at pH 7.5, 6.5, and 5, and containing or not 0.8 M trehalose. Moreover, the observed DLS peaks were further inspected via their deconvolution into Gaussian components (using the Fityk software [39]) as we previously carried out for rabbit and human LDH-A [34,42]. By this means, we evaluated the partition of the enzyme ensemble into sub-populations representing tetramer, dimer, and two monomeric forms [34,42]. When samples of rabbit LDH-A exposed to pH 7.5 were considered, no major differences were detected between enzyme samples containing trehalose or devoid of the disaccharide (Fig.s 5A and 5 B). In particular, in the absence of trehalose we detected LDH-A forms featuring a diameter equal to 12.62, 10.08, 8.27, and 6.96 nm (Fig. 5A), which we relate to enzyme tetramer, dimer, and two monomeric forms, respectively. Interestingly, in the presence of trehalose we identified by deconvolution diameters equal to 11.91, 9.85, 8.42, and 7.39, respectively (Fig. 5B). It is also important to note that the relative area of the different peaks composing the enzyme population are not significantly affected by the addition of trehalose to LDH-A (Table 2). Interestingly, when the enzyme was exposed to pH 6.5 in the absence of trehalose the diameters of the different enzyme forms were calculated as equal to 12.67, 10.50, 8.87, and 7.71 nm (Fig. 5C), in good agreement with the diameter values estimated at pH 7.5 under the same conditions. However, it is important to note that the relative abundance of tetramer and dimer (as suggested by the corresponding peak areas, Table 2) in the absence of trehalose were respectively negatively and positively affected by lowering the pH from 7.5 to 6.5, suggesting a pH-induced dissociation of LDH-A tetramer into dimers. Conversely, when trehalose was present in the enzyme solution, the peak areas of the tetramer, the dimer, and the monomers were not significantly different when comparing samples poised at pH 7.5 or 6.5 (Fig. 5D, Table 2). Even more surprising were the observations obtained with samples prepared at pH 5. Indeed, in the absence of trehalose we did not detect LDH-A tetramer, with the enzyme ensemble dominated by dimer and by one of the two monomeric forms (Fig. 5E, Table 2). On the contrary, the addition of trehalose to LDH-A exposed to pH 5 completely counteracted the dissociation of enzyme induced by the acidic pH (Fig. 5F, Table 2). It should indeed be noted that trehalose was effective in maintaining invariant the relative abundance of LDH-A tetramer, dimer and monomers in solutions poised at pH 7.5, 6.5, and 5 (Table 2).

3.4. Analysis by mass spectrometry of tetrameric LDH-A dissociation

The dissociation of tetrameric rabbit LDH-A was also investigated by mass spectrometry. Surprisingly, we detected a consistent dissociation of the tetrameric enzyme even under conditions of neutral pH (Fig. 6A). In particular, tetrameric and dimeric forms of LDH-A were clearly identified over the 5000–6000 and the 2200–4300 interval of the *m/z* values of the mass spectrum, respectively (Fig. 6A, Tab. ST1). The calculated molecular mass of these forms was estimated as equal to 146203.4302 ± 0.5845 and 73110.8392 ± 0.9708 for LDH-A tetramer and dimer, respectively (Tab. ST1). In addition, we also observed a monomeric form characterized by positive charged states ranging from +10 to +16, and featuring a molecular mass estimated equal to 36564.414 ± 0.556 (Fig. 6A, Tab. ST1). Quite intriguingly, the

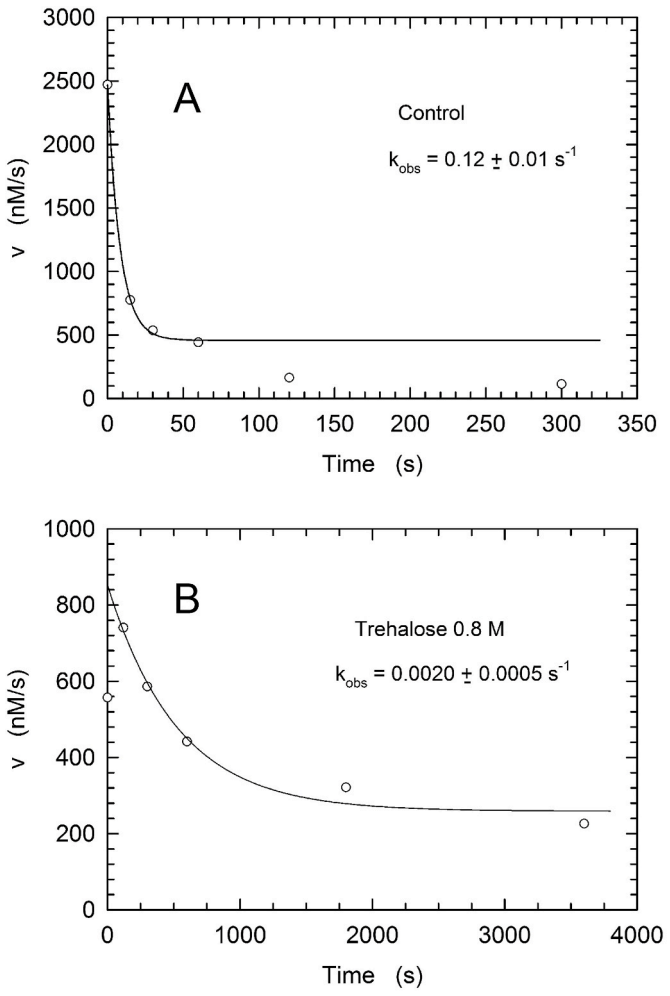


Fig. 4. Stability of rabbit LDH-A at pH 5.
The activity of 10.8 nM tetrameric rabbit LDH-A (43.2 nM subunits) was determined as a function of the enzyme residence time at pH 5. Reaction mixtures contained 125 μM β-NADH and 500 μM pyruvate. The assays were performed in the absence (**A**) or in the presence of 0.8 M trehalose (**B**).

inspection of the mass spectrum revealed the presence of additional components featuring a limited number of charged states (from +10 to +13), and whose molar mass is compatible with that of the LDH-A monomer (Fig. 6A, Tab. ST1). Further, a similar pattern was observed when a sample of LDH-A at pH 5 was analyzed (Fig. 6B, Tab. ST2). Remarkably, it has been previously demonstrated that ESI mass spectrometry (ESI-MS) is a suitable technique to discriminate native and denatured conformations of proteins. In particular, when cytochrome *c* and lysozyme were analyzed by ESI-MS, their native conformers generated peaks featuring charged states from +7 to +9 and from +9 to +11, respectively [43,44]. Moreover, when both proteins were subjected to full denaturation, peaks with charged states ranging from +10 to +20 (cytochrome *c*) and from +14 to +22 (lysozyme) were detected [43,44]. According to these observations, we propose that the LDH-A monomer identified by the +10/+16 and by the +10/+13 ESI multicharged envelopes correspond to the fully and partially denatured enzyme, respectively. This conformational complexity revealed by the ESI-MS analysis of monomeric rabbit LDH-A is most likely mirrored in our DLS experiments, according to which we detected two major forms of the monomeric enzyme.

3.5. Effect of maltose and trehalose on reactions catalyzed by different LDH-A concentrations at pH 5

The catalytic action of rabbit LDH-A is known to feature a peculiar dependence on enzyme concentration [45]. When the activity of rabbit LDH-A exerted at the expense of β-NADH and pyruvate was assayed at pH 7.4 in the presence of ca. 2 μg/mL of enzyme, the reaction velocity was found to be approximately equal to 0.6 mM/s•mg [45]. However, when the concentration of enzyme in the assay was lowered to ca. 1 μg/mL, pyruvate reduction was hardly detectable, and turned extremely modest, if at all, in the presence of enzyme at concentrations slightly lower than 1 μg/mL [45]. These findings suggest a high propensity of rabbit LDH-A to undergo dilution-induced dissociation. It was indeed shown that when the enzyme is diluted at concentrations lower than 1 mg/mL (at pH 7.0) significant dissociation occurs, albeit at a slow rate [46]. Moreover, it was reported that LDH-A at 0.1 mg/mL is equally partitioned into tetramer and dimer + monomer, with further dilution to 0.05 mg/mL inducing a considerable dissociation of the tetramer, accounting for 10% only of the total enzyme [47]. Notably, it was also shown that dissociated and immobilized rabbit LDH-A is able to reassemble with free subunits, albeit this competence is rapidly lost [35].

It is important to note here that the susceptibility of tetrameric rabbit LDH-A to dissociate when subjected to dilution can be enhanced by acidic pH, as we have previously shown with DLS experiments [34]. Accordingly, we decided to perform activity assays under conditions strongly favoring tetrameric LDH-A dissociation, i.e. at pH 5 and in the presence of different enzyme concentrations. In particular, reaction mixtures were composed of 100 μM β-NADH, 0.5 mM pyruvate, and were supplemented with maltose (the presence of which at pH 5 does strongly inhibit LDH-A activity, cf. Fig. 1C) or trehalose. Control samples not containing any disaccharide were also considered. When the reduction of pyruvate was assayed in the presence of 7.5 nM tetrameric LDH-A the observed kinetics was rather fast, leading to the attainment of equilibrium in a few minutes (Fig. 7A). Strikingly, a very similar kinetics was observed when the reaction mixture was supplemented with 30 mM maltose (Fig. 7A). However, when 5.25 nM enzyme was used, the control reaction was found to be much slower when compared with that detected in the presence of 7.5 nM enzyme (Fig. 7B). This dilution-related effect was strongly enhanced by maltose, the presence of which did largely reduce the amplitude of β-NADH oxidation over the first 20 min of reaction (Fig. 7B). We propose that this action exerted by maltose is due to its competence in binding the dissociated forms of rabbit LDH-A, with this binding being responsible for the stabilization of the enzyme dissociated forms. When the concentration of enzyme in the assay mixture was further lowered to 3.75 nM, the oxidation of 30% of the β-NADH initial concentration occurred over a quite long time interval, i.e. 100 min (Fig. 7C). Notably, the presence of maltose slowed further down the reaction, and was responsible for suppressing an initial fast phase of β-NADH oxidation (Fig. 7C). We then tested the effects exerted by trehalose, at 0.4 or 0.8 M final concentration, on reaction kinetics. When assays were performed over 10 min in the presence of 7.5 nM tetrameric LDH-A, the addition to the assay mixture of 0.4 or 0.8 M trehalose induced an increased and a decreased amplitude of β-NADH oxidation, respectively (Fig. 8A). In addition to this effect, trehalose did significantly decrease the initial reaction velocity, with the higher concentration corresponding to the more pronounced outcome (Fig. 8A). Further, quite surprising observations were obtained in the presence of 5.25 nM enzyme. Indeed, the addition of trehalose, both at 0.4 and 0.8 M, was responsible for leading the reaction to completion in approximately 20 min, with the control reaction reaching 30% only of β-NADH conversion over the same time-interval (Fig. 8B). Finally, we observed that trehalose was able to strongly favor the action of 3.75 nM enzyme, with 0.8 M of the disaccharide prompting the attainment of equilibrium within ca. 100 min (Fig. 8C). It is also important to note that a considerable improvement of the enzyme catalytic action was triggered by 0.4 M trehalose, albeit to a lower extent when compared to that

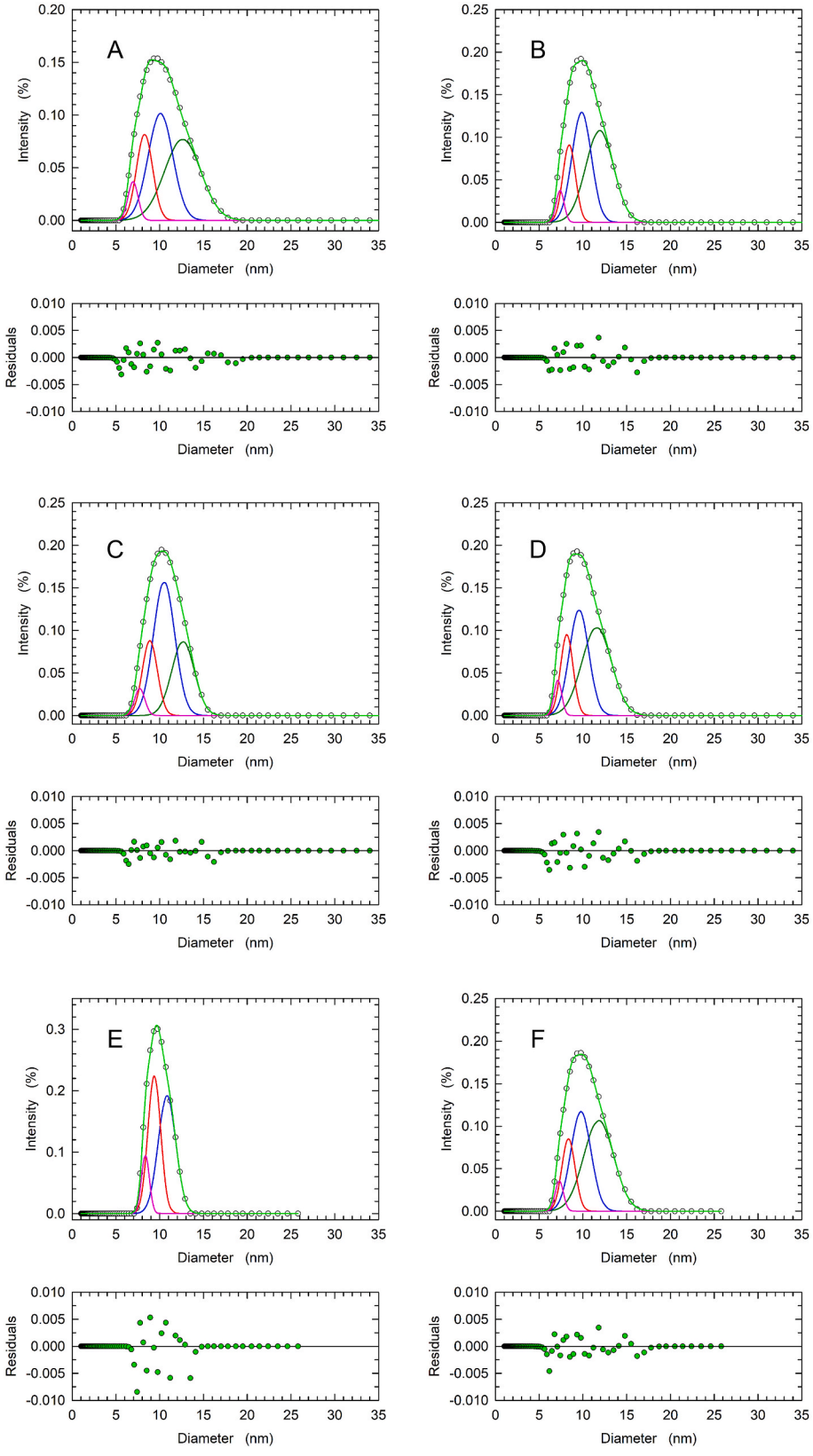


Fig. 5. Effect of pH and of trehalose on the relative abundance of rabbit LDH-A association forms.
Dynamic light scattering experiments were performed in the presence of 15.4 μM tetrameric LDH-A (61.6 μM subunits) at pH 7.5 (**A,B**), 6.5 (**C,D**), and 5.0 (**E, F**). The enzyme samples were devoid of any disaccharide (A, C, and E) or contained 0.8 mM trehalose (B, D, and F). The distribution of the observed intensities of scattering (empty circles) were deconvoluted with the Fityk software into an ensemble of Gaussian components (dark green, blue, red, and pink lines). The areas of the peaks to be deconvoluted were normalized to 1. The fittings to the experimental observations accordingly obtained are reported with green lines.

observed in the presence of 0.8 M of the same disaccharide (Fig. 8C).

Overall, these observations indicate that trehalose effectively counteracts the dissociation of tetrameric LDH-A at pH 5, with the preservation of enzyme activity being effectively induced by 0.8 M trehalose.

3.6. Modeling of the reactions catalyzed by LDH-A at pH 5

To interpret the experimental observations obtained at pH 5 as a function of rabbit LDH-A concentration (Fig.s 7 and 8), we devised a model which takes into account how enzyme dissociation affects

Table 2
Dynamic light scattering analysis of rabbit LDH-A association forms.
The diameters of different rabbit LDH-A oligomers were estimated by deconvolution of the peaks observed with dynamic light scattering experiments in the presence of 15.4 μM enzyme (61.6 μM subunits), at pH 7.5, 6.5, and 5.0. The area of each peak to be deconvoluted was normalized to 1, and the relative abundance of each component identified by deconvolution is accordingly indicated. T, D, and M denote the assignment of a detected component to enzyme tetramer, dimer, and monomer, respectively.

Control	Diameter (nm)	Area (%)
pH 7.5 15.4 μM LDH (Holo)	12.62 (T)	40.1
	10.08 (D)	35.9
	8.27 (M)	18.9
	6.96 (M)	5.3
pH 6.5 15.4 μM LDH (Holo)	12.67 (T)	28.2
	10.50 (D)	48.3
	8.87 (M)	19.0
	7.71 (M)	4.7
pH 5.0 15.4 μM LDH (Holo)	10.83 (D)	48.6
	9.38 (M)	41.2
	8.36 (M)	10.8
Trehalose (0.8 M)	**Diameter (nm)**	**Area (%)**
pH 7.5 15.4 μM LDH (Holo)	11.91 (T)	43.3
	9.85 (D)	36.0
	8.42 (M)	16.7
	7.39 (M)	4.3
pH 6.5 15.4 μM LDH (Holo)	11.57 (T)	44.0
	9.56 (D)	34.5
	8.16 (M)	17.1
	7.14 (M)	4.6
pH 5.0 15.4 μM LDH (Holo)	11.80 (T)	46.1
	9.77 (D)	33.8
	8.34 (M)	16.2
	7.30 (M)	4.2

reaction kinetics (see Methods). It should be noted that to contain the number of rate constants whose numerical values were let free to fluctuate we imposed a couple of constraints to the model: i) the k_1/k_{-1} ratio was constrained to be equal to 1, in agreement with the value determined at pH 6 by means of the T-jump technique for pig heart LDH [48]; ii) considering that the dissociated forms of rabbit LDH-A are prone to aggregation at pH 5 [34], the k_4/k_{-4} ratio was kept constant and equal to 10^4. In the absence of any disaccharide, the pseudo-first-order rate constant k_{on} (related to the step generating the reaction products) was found to decrease as the total enzyme concentration was lowered (Fig. 9, Table 3). We propose that this mutual relation represents the outcome of dissociation-reassociation events of tetrameric LDH-A, leading to an unfavorable conformational heterogeneity of the enzyme. In particular, the dilution of rabbit LDH-A appears to decrease the frequency of its dissociation-reassociation (as suggested by the values of the rate constants k_3 and k_{-3}), therefore inducing a decreased sampling of the conformational landscape. It should also be noted that the value of the k_3/k_{-3} ratio is inversely proportional to the initial total enzyme concentration, indicating a predominance of enzyme dissociation under diluted conditions. When the LDH-A action in the presence of maltose is considered, the dependence on enzyme concentration of the k_{on} and k_{off} rate constants was found to resemble the corresponding relation determined in the absence of the disaccharide (Fig. 9, Table 3). However, it should be noted that the addition of maltose to reaction mixtures containing low enzyme concentrations induced an increase of the k_3/k_{-3} ratio (Table 3). Accordingly, maltose appears to favor the dissociation of rabbit LDH-A tetramer, therefore triggering a significant decrease of reaction velocity. When reaction mixtures were supplemented with 0.4 or 0.8 M trehalose a peculiar effect was observed. In particular, the presence of trehalose was found to significantly decrease the value of k_{on} estimated in the presence of 7.5 nM enzyme (Fig. 10, Table 3). Nevertheless, upon dilution of rabbit LDH-A, k_{on} was found to undergo a moderate decrease when compared to the corresponding k_{on} values determined for control reactions and for assays carried out in the

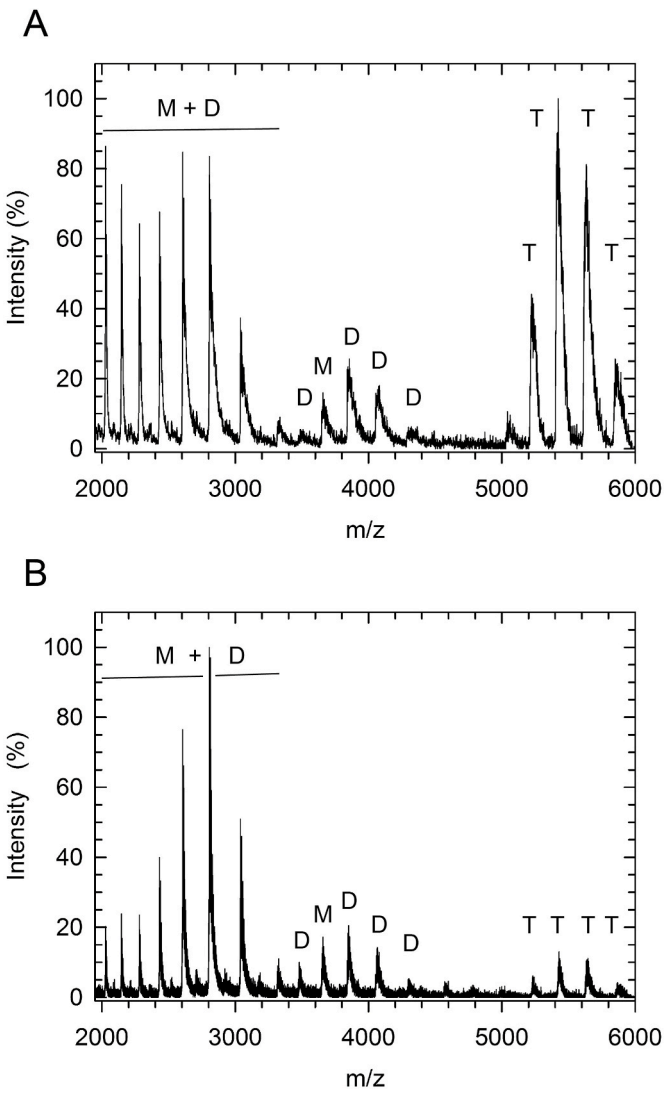
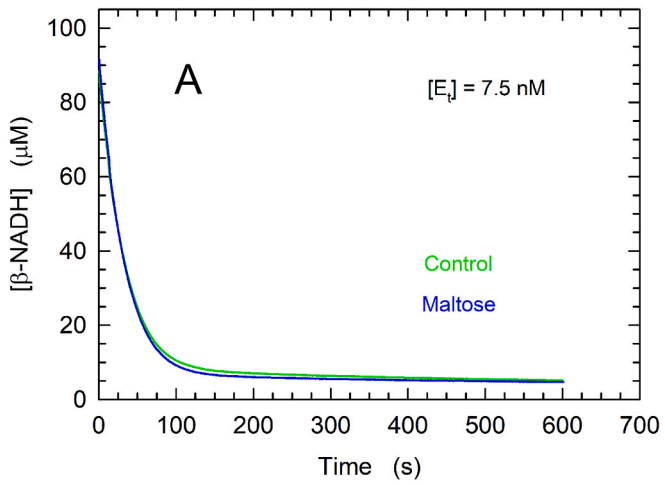


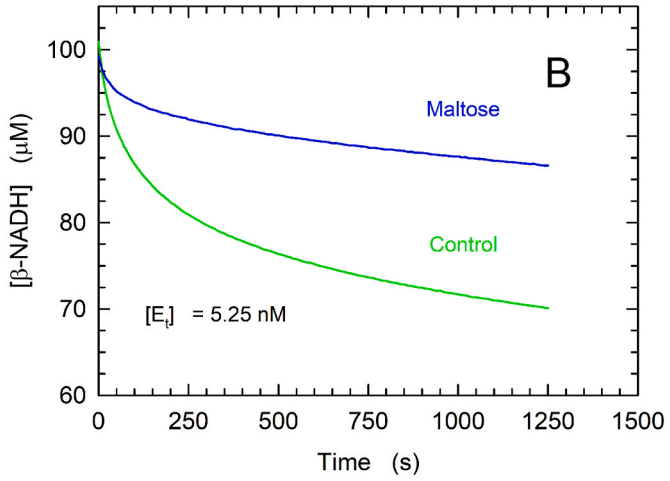
Fig. 6. ESI mass spectra of rabbit LDH-A samples poised at pH 5 and 7.
Mass spectra of 4 μM tetrameric LDH-A (16 μM subunits) poised at pH 7 (**A**) or 5 (**B**) using a buffer containing ammonium acetate and ammonium bicarbonate. The peaks generated by the enzyme tetramer, dimer and monomer are labeled T, D, and M, respectively.

presence of maltose (Fig. 10, Table 3). We interpret this complex behavior by taking into account that the reaction media containing trehalose feature a rather high viscosity, which limits the enzyme conformational dynamics and therefore its catalytic performance. Further, trehalose appears to limit enzyme dissociation, as suggested by the values of the k_3/k_{-3} ratio determined in the presence of this disaccharide (Table 3). In addition, it should be noted that the magnitude of the values estimated for the k_3 and k_{-3} rate constants are significantly lower when compared to those determined in the presence of maltose or in the absence of any disaccharide (Table 3). This suggests that trehalose promotes an enzyme conformation less susceptible to dissociation and featuring limited dynamics, containing in turn its catalytic action.

4. Discussion

Different models were proposed to explain how trehalose enhances protein stability, and particular attention was devoted to proteins hosted in low-water trehalose matrices. Concerning the stability of membrane proteins, an important factor to consider is the dehydration-rehydration of the protein-membrane assembly. It is indeed known that the





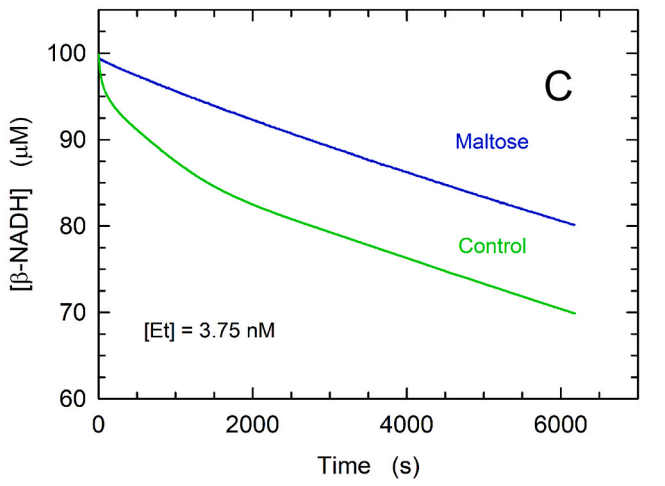
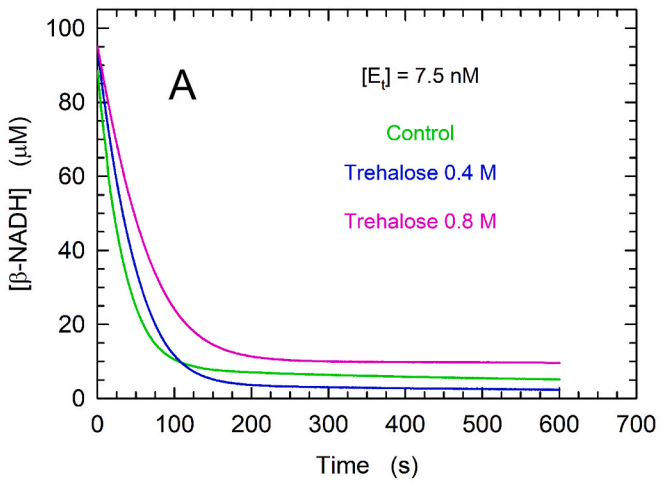
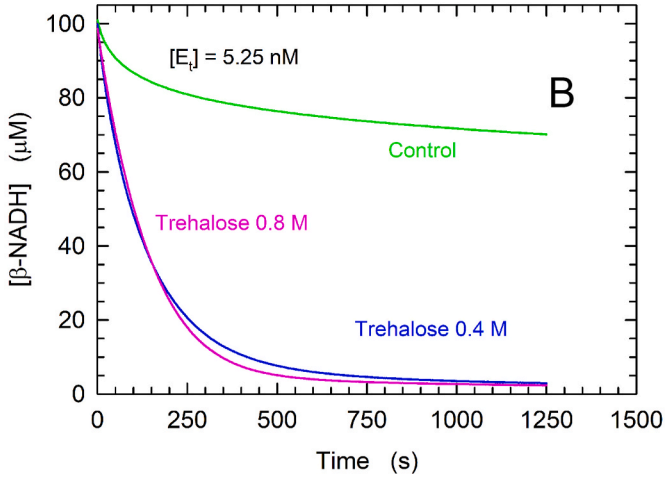


Fig. 7. Kinetics of pyruvate reduction catalyzed by rabbit LDH-A in the absence or in the presence of maltose.
The activity of rabbit LDH-A was assayed in the presence of 100 μM β-NADH, 500 μM pyruvate and 7.5 (**A**), 5.25 (**B**), or 3.75 (**C**) nM tetrameric enzyme. Reaction mixtures were devoid of any disaccharide (green lines) or were supplemented with 30 mM maltose (blue lines).





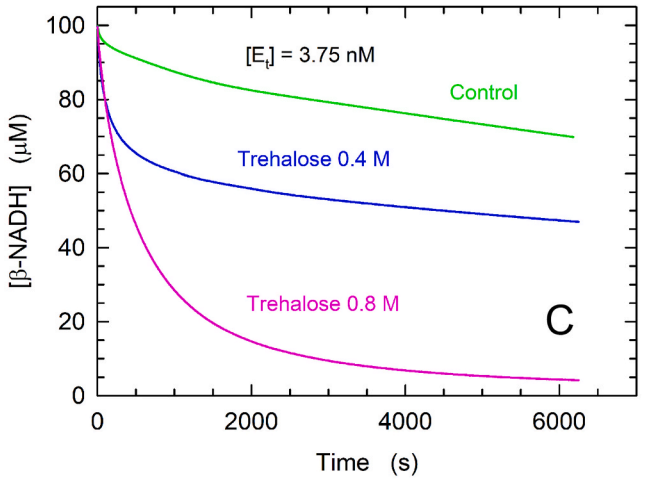


Fig. 8. Kinetics of pyruvate reduction catalyzed by rabbit LDH-A in the absence or in the presence of trehalose.
The activity of rabbit LDH-A was assayed in the presence of 100 μM β-NADH, 500 μM pyruvate and 7.5 (**A**), 5.25 (**B**), or 3.75 (**C**) nM tetrameric enzyme. Reaction mixtures were devoid of any disaccharide (green lines) or were supplemented with 0.4 M (blue lines) or 0.8 M (pink lines) trehalose.

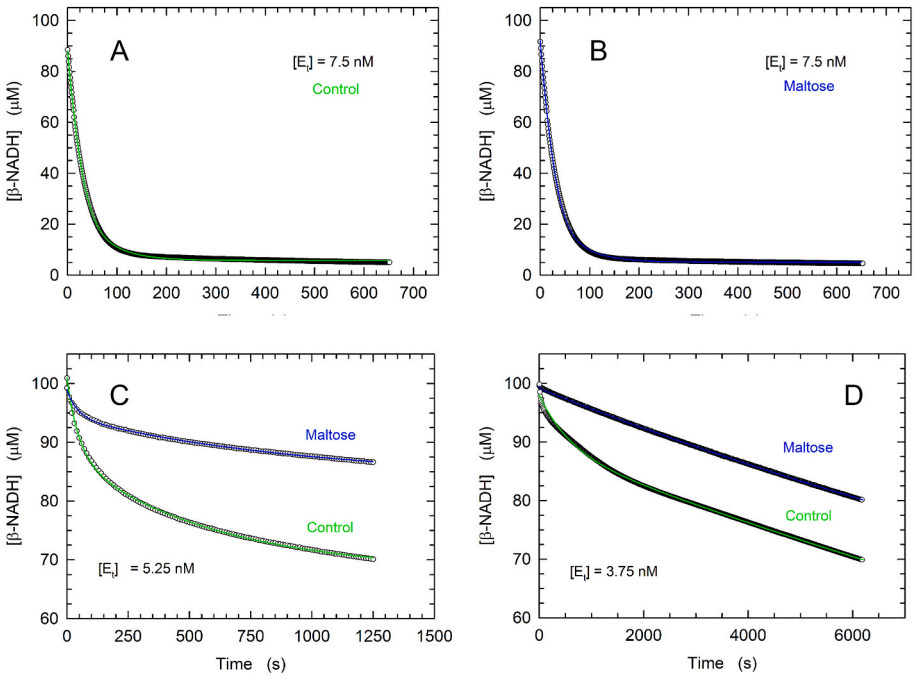


Fig. 9. Observed and simulated kinetics of pyruvate reduction catalyzed by rabbit LDH-A in the absence or in the presence of maltose.
(**A**) The time-course of β-NADH oxidation coupled to the reduction of pyruvate catalyzed by 7.5 nM tetrameric LDH-A is shown with empty circles, with the corresponding simulated kinetics (see Methods) reported with a green line. The experimental observations are the same of Fig. 7A. (**B**) Observed (empty circles) and simulated (blue line) kinetics of β-NADH oxidation coupled to the reduction of pyruvate catalyzed by 7.5 nM tetrameric LDH-A in the presence of 30 mM maltose. The experimental observations are the same shown in Fig. 7A. (**C,D**) Observed (empty circles) and simulated kinetics (continuous lines) of β-NADH oxidation coupled to the reduction of pyruvate catalyzed by 5.25 (C) or 3.75 (D) nM tetrameric LDH-A in the absence (green lines) or in the presence (blue lines) of 30 mM maltose. The experimental observations are the same shown in Fig.s 7B and 7C.

Table 3
Numerical values of the rate constants estimated by simulating the kinetics of the reaction catalyzed by rabbit LDH-A.
The observed time-course of β-NADH oxidation coupled to the reduction of pyruvate catalyzed by different concentrations of rabbit LDH-A was interpreted with a kinetic model (see Methods) used to estimate the numerical values of the rate constants associated to the reversible steps leading to product generation (k_{on} and k_{off}) and enzyme dissociation (k_3 and k_{-3}).

Control				
[E]	k_{on}	k_{off}	k_3	k_{-3}
7.50 nM	295 ± 1	20.5 ± 0.1	1420 ± 50	305000 ± 9000
5.25 nM	62 ± 1	127 ± 1	1.38 ± 0.02	37 ± 1
3.75 nM	5.06 ± 0.02	0.02 ± 0.01	0.133 ± 0.001	3.28 ± 0.01
Maltose				
[E]	k_{on}	k_{off}	k_3	k_{-3}
7.50 nM	321 ± 1	19 ± 1	14300 ± 400	(3.0 ± 0.1)•10^6
5.25 nM	24.1 ± 0.2	133 ± 2	2.06 ± 0.03	33 ± 1
3.75 nM	6.3 ± 0.4	3.1 ± 0.3	18 ± 2	330 ± 30
Trehalose 0.4 M				
[E]	k_{on}	k_{off}	k_3	k_{-3}
7.50 nM	215.7 ± 0.2	6.11 ± 0.02	22.2 ± 0.4	5380 ± 80
5.25 nM	147.7 ± 0.2	5.12 ± 0.02	1.043 ± 0.006	136 ± 1
3.75 nM	64.1 ± 0.2	55.2 ± 0.2	0.357 ± 0.001	4.00 ± 0.03
Trehalose 0.8 M				
[E]	k_{on}	k_{off}	k_3	k_{-3}
7.50 nM	158.2 ± 0.1	17.98 ± 0.01	7.3 ± 0.1	2350 ± 30
5.25 nM	133.1 ± 0.1	3.68 ± 0.01	1.51 ± 0.01	283 ± 2
3.75 nM	54.3 ± 0.1	2.69 ± 0.02	0.2022 ± 0.0009	10.9 ± 0.1

dehydration of membranes featuring a gel state yields low-water samples whose rehydration translates into a consistent destabilization. However, trehalose was shown to prevent gel state formation during the dehydration of membranes by replacing water molecules at the interface between phospholipids and the bulk solvent [49,50]. Accordingly, this mechanism has been denoted as the water replacement hypothesis (WRH) [49]. In addition to WRH, the competence of trehalose in stabilizing membranes and dehydrated proteins has been explained with alternative models. In particular, and as already mentioned here, the high T_g of trehalose is considered a major factor responsible for the preservation of dehydrated bacteria, liposomes, and proteins [10–16]. Mechanistically speaking, the protection exerted by trehalose on dehydrated proteins has been related to the exclusion of the disaccharide from the surface of proteins (preferential exclusion hypothesis), therefore preserving their hydration shell [51]. Further, the glassy nature of trehalose matrices would be responsible for trapping the water layer (water-entrapment hypothesis) around the surface of dehydrated proteins [52].

Trehalose is not only able to stabilize proteins under low-water conditions, but it does also increase the stability of proteins in aqueous solutions [21–26]. As a further inspection of this action, we have reported here on the capability of trehalose to prevent the dissociation of an oligomeric enzyme, using rabbit skeletal muscle lactate dehydrogenase as a model system. We indeed previously reported that this tetrameric enzyme is subjected to dissociation when exposed to acidic aqueous solutions [34].

First, we compared the effect, if any, exerted by cellobiose, maltose and trehalose on the activity of LDH-A determined at different pH values, i.e. 5, 6, 6.5, and 7.5. Quite surprisingly, we observed that the addition of cellobiose or maltose to reaction mixtures poised at pH 5 triggered a sharp inhibition of enzyme activity (Fig. 1). On the contrary, trehalose induced a notable enhancement of LDH-A activity at pH 5, with an exponential dependence of catalytic action on trehalose concentration. Notably, major effects were observed in the presence of 0.6–0.9 M trehalose (Fig. 2). This is in agreement with observations reported by Mamedov et al., indicating that the rate of oxygen evolution by Photosystem II (PS-II) core complexes and by membrane fragments containing PS-II was strongly enhanced by 0.5 or 1 M trehalose [53,54].

The observation that cellobiose and maltose exert an antagonistic action toward rabbit LDH-A whereas trehalose stabilizes this enzyme is rather intriguing. Among the reasons responsible for these opposed effects, it could be of importance that cellobiose and maltose are reducing disaccharides whereas trehalose is not. This, in turn, implies that cellobiose and maltose can adopt an aldehydic open form, in contrast to the non-reducing trehalose. Remarkably, it was proposed that the opening of the glucose ring can be catalyzed by protonated lysines, e.g. by lysine 199 of human serum albumin [55]. Accordingly, we propose that the glucose moiety of cellobiose and maltose is subjected to an

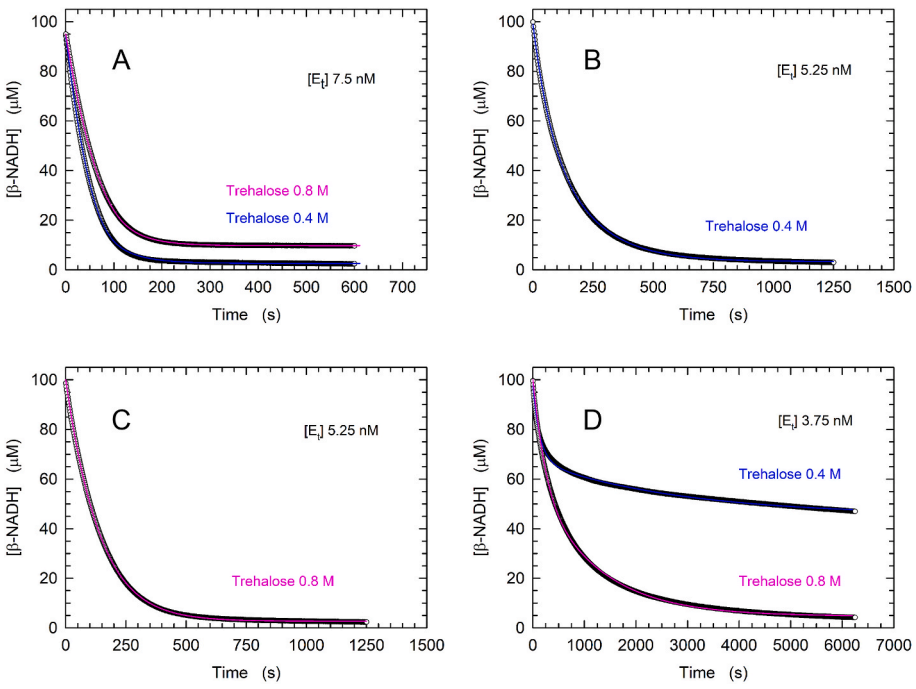


Fig. 10. Observed and simulated kinetics of pyruvate reduction catalyzed by rabbit LDH-A in the presence of trehalose. (**A**) The time-course of β-NADH oxidation coupled to the reduction of pyruvate catalyzed by 7.5 nM tetrameric LDH-A in the presence of 0.4 or 0.8 M trehalose is shown with empty circles, with the corresponding simulated kinetics (see Methods) reported with blue (0.4 M trehalose) and magenta (0.8 M trehalose) lines. The experimental observations are the same of Fig. 8A. (**B**) Observed (empty circles) and simulated (blue line) kinetics of β-NADH oxidation coupled to the reduction of pyruvate catalyzed by 5.25 nM tetrameric LDH-A in the presence of 0.4 M trehalose. The experimental observations are the same shown in Fig. 8B. (**C**) Observed (empty circles) and simulated kinetics (magenta line) of β-NADH oxidation coupled to the reduction of pyruvate catalyzed by 5.25 nM tetrameric LDH-A in the presence of 0.8 M trehalose. The experimental observations are the same shown in Fig. 8B. (**D**) Observed (empty circles) and simulated (continuous lines) kinetics of β-NADH oxidation coupled to the reduction of pyruvate catalyzed by 3.75 nM tetrameric LDH-A in the presence of 0.4 (blue line) or 0.8 M (magenta line) trehalose. The experimental observations are the same of Fig. 8C.

analogous mechanism when buried lysines of rabbit LDH-A (e.g. K41) are let, as a result of enzyme dissociation, to interact and to open the glucose ring of these disaccharides. We also propose that these cellobiose- and maltose-specific interactions could lead to the stabilization of the dissociated forms of rabbit LDH-A.

Further, we determined the kinetic parameters of LDH-A at pH 5, 6.5, and 7.5, in the absence or in the presence of trehalose (0.4 or 0.8 M). Not surprisingly, at pH 7.5 trehalose triggered a decrease of both the K_m for pyruvate and V_{max} (Table 1), as it was previously reported for rabbit LDH-A assayed at 25 °C and under neutral pH conditions [33]. Moreover, our observations do quantitatively agree with those obtained by Hernández-Meza and Sampedro [33], indicating that the addition of 0.8 M trehalose to reaction mixtures is responsible for halving the values of both V_{max} and K_m (Table 1, [33]). Interestingly, it was proposed that this effect stems from the viscosity of aqueous solutions containing trehalose, suggesting that viscosity would induce: i) a slower conformational dynamics of LDH-A, which would in turn negatively affect the maximal rate of the enzyme-catalyzed reaction [33]; ii) a decrease of the motions of an intrinsically disordered loop which is engaged in pyruvate binding through histidine 192, with these decreased motions promoting a favorable conformation for substrate binding [33]. Generally speaking, the effect of viscosity on enzyme catalysis can be interpreted according to Kramers' theory of reaction kinetics in liquid systems [56]. In particular, when diffusion is rate-limiting an enzyme-catalyzed reaction, Kramers' theory designates the friction between reactants and solvent as a major regulation exerted by viscosity on reaction velocity. Remarkably, both the conformational dynamics and the folding rate of proteins can be considered diffusional processes significantly affected by viscosity [57,58].

In addition to the assays at pH 7.5, we also inspected the activity of LDH-A at pH 6.5 and 5, i.e. under acidic conditions inducing a transition from Michaelis-Menten to sigmoidal kinetics [34]. Surprisingly, we observed that at pH 6.5 trehalose elicited a less pronounced decrease of V_{max} and K_m when compared to the effect detected at pH 7.5. Moreover, at pH 6.5 the Hill coefficient was not affected by the presence of 0.4 or 0.8 M trehalose (Table 1). Therefore, it is important to mention here that we have previously interpreted the sigmoidal kinetics featured by LDH-A under acidic conditions as the output of allosteric transitions related to dissociation-association equilibria of the tetrameric enzyme [34]. Specifically, we proposed this interpretation taking into account the allosteric models devised by Nichol et al. and by Frieden [59,60]. Accordingly, our observations at pH 6.5 suggest that trehalose counteracts, albeit not completely, the dissociation of rabbit LDH-A, the assembly of which is favoured by the homotropic action of pyruvate (Fig. 3). By performing activity assays at pH 5, we observed quite striking effects generated by trehalose. Besides the unsurprising decrease of K_m, we indeed detected a considerable increase of V_{max} when trehalose was added to reaction mixtures. In particular, in the presence of 0.4 M trehalose LDH-A featured: i) sigmoidal kinetics, and a value of the Hill coefficient not significantly different when compared to that determined in the absence of trehalose; ii) a 2.5-fold increase of V_{max}. In addition, very high enzyme activity was observed at pH 5 in the presence of 0.8 M trehalose. Moreover, under these conditions we clearly detected substrate inhibition at pyruvate concentrations >250 μM and a very sharp increase of the initial reaction velocities at substrate concentrations ranging from 20 to 100 μM. This very sharp increase hampers a reliable interpretation of the observed kinetics according to the Michaelis-Menten or to an allosteric model. However, to qualitatively compare these data with those obtained at pH 6.5 we fitted the Michaelis-Menten equation to the experimental observations obtained at pH 5 in the presence of 0.8 M trehalose. By this means, the estimated V_{max} was almost 3 times higher when compared to the value obtained in the presence of 0.4 M trehalose (Fig. 3F, Table 1, and Fig. S1). Accordingly, the ensemble of the kinetic data obtained at pH 5 suggests that trehalose does very efficiently inhibit LDH-A dissociation. To further inspect this point, we evaluated the decay rate of LDH-A activity as a function of the residence time imposed to the enzyme at pH 5, and we analyzed the distribution of the different associated/dissociated enzyme forms as a function of pH. By performing kinetic and DLS experiments, we found that 0.8 M trehalose decreases 60-times the rate constant related to the decay of LDH-A activity (Fig. 4), and does strongly inhibit enzyme dissociation (Fig. 5). Remarkably, the diameters estimated by DLS for the different associated and dissociated forms of LDH-A appear to be consistent. Indeed, taking into account all the values estimated in the absence of trehalose (Table 2) we indeed obtained 12.7 ± 0.1, 10.5 ± 0.4, 8.8 ± 0.6, and 7.7 ± 0.7 nm for the diameter of tetramer, dimer, and the two monomeric forms, respectively. Moreover, in the presence of 0.8 M trehalose the estimated diameter values were equal to 11.8 ±

0.2, 9.7 ± 0.2, 8.3 ± 0.1, and 7.3 ± 0.1 nm for tetramer, dimer, and for the two monomeric forms, respectively. Therefore, not only the identification of the different enzyme forms appears reliable, but we have also revealed that trehalose triggers a slight but significant decrease of the diameter of tetrameric and dimeric LDH-A. This finding is in agreement with observations obtained by DLS using bovine serum albumin (BSA). It was indeed determined that BSA undergoes a considerable shrinkage when exposed to 0.5 M trehalose, with the protein diameter decreasing from 9.69 to 6.59 nm [60]. This contraction of BSA, as well as that reported here for LDH-A, is most likely related to a thinner hydration shell featured by proteins residing in trehalose solutions instead of being hosted in environments devoid of the disaccharide. Moreover, it is important to note that the shrinkage of BSA elicited by trehalose is independent of the viscosity conferred by this disaccharide to aqueous solutions. Indeed, when BSA was exposed to a solution containing 20% glycerol (i.e. to an environment featuring a similar viscosity to that possessed by aqueous 0.5 M trehalose) the protein diameter was determined as equal to 17.24 nm [61].

To further unveil the action of trehalose against the dissociation of tetrameric LDH-A, we performed activity assays at pH 5 as a function of enzyme concentration. Further, we also considered the action of maltose, and we interpreted the observed reaction kinetics using a model which takes into account enzyme dissociation (by means of the two rate constants k_3 and k_{-3}, see Methods). Interestingly the values obtained for the k_{-3}/k_3 ratio (Fig. S2) indicate that: i) maltose favors the dilution-induced enzyme dissociation; ii) the presence of 0.4 M trehalose counteracts dissociation, except when enzyme was diluted to 3.75 nM; iii) the addition of 0.8 M trehalose to reaction mixtures did strongly inhibit LDH-A dissociation, independently of the particular enzyme dilution considered. It should be noted that the enhancement of LDH-A activity triggered by trehalose at pH 5 is counteracted by the viscosity conferred by this disaccharide to the reaction milieu. Therefore, it is conceivable that both the stimulation of LDH-A activity by 0.8 M trehalose (as reported here) and the enhancement by trehalose of the PSII oxygen-evolving activity [53,54] are most presumably underestimated. Interestingly, it was demonstrated that trehalose is engaged in interactions with the donor side of PS-II [54]. Further, it was proposed that trehalose promotes the maintenance of a PS-II conformation favorable for the release of reaction products [54]. In addition to this, and in line with the observations reported here, it can be suggested that trehalose hampers the dissociation of dimeric PS-II core. It was indeed shown that the PS-II core complex can be isolated as a monomer or as a dimer, with molecular masses estimated equal to 236 and 430 kDa, respectively [62]. Moreover, it was demonstrated that dimeric PS-II core performs its catalytic action more efficiently than its monomeric counterpart [62]. Accordingly, the inhibition of PS-II core dissociation by trehalose would translate into a higher level of oxygen evolution.

5. Concluding remarks

The observations reported here indicate that trehalose counteracts the pH-induced dissociation of tetrameric rabbit skeletal muscle lactate dehydrogenase. Notably, this finding expands the repertoire of ways according to which trehalose enhances protein stability. It will therefore be of interest to test whether this disaccharide antagonizes or not the dissociation of other oligomeric enzymes. To this aim, dehydrogenases appear as suitable candidates when taking into account their sensitivity to dissociation affected by acidic conditions. Finally, it is our hope that the enzymatic production of trehalose from starch [63] will contribute to contain the cost of this intriguing disaccharide, enabling its use for the stabilization of industrial enzymes.

Authorship contribution statement

Michelangelo Simongini, Andrea Puglisi: Investigation. Filippo Genovese: Investigation (mass spectrometry experiments). Alejandro Hochkoeppler: conceptualization, supervision, writing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.abb.2023.109584.

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