Master's Program
»Physics of Life«
»Computational Life Science«

Lab Course:
» Molecular Biology and Biochemistry of Life«

Topics: Characterization of protein stability, catalysis and aggregation

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Contents

Timetable

The time table for each group will be made available through OPAL

Times

Morning: 9 am-12 pm

Afternoon: 1 pm – 4 pm

Devices

Chem. unf.: Prometheus Panta (NanoTemper)

Enzyme: NP80 (Implen)

Aggregation: Spark Plate Reader (Tecan)

Temp: Prometheus Panta (NanoTemper)

Protein folding and stability

Protein folding is the physical process by which a protein chain acquires its native 3-dimensional structure, a conformation that is usually biologically functional. Each protein exists as an unfolded polypeptide when translated from a sequence of mRNA to a linear chain of amino acids. As the polypeptide chain is being synthesized by a ribosome, the linear chain begins to fold into its three-dimensional structure. The resulting three-dimensional structure is determined by the amino acid sequence or primary structure.

The correct three-dimensional structure is essential to function (biologically active form = native structure, native conformation), although some parts of functional proteins may remain unfolded. Failure to fold into native structure generally produces inactive proteins, but in some instances misfolded proteins have modified or toxic functionality. Several neurodegenerative and other diseases are believed to result from the accumulation of amyloid fibrils formed by misfolded proteins.

Denaturation of proteins is a transition of the folded to the unfolded state. It happens in cooking, in burns, as well as in proteinopathies (protein folding diseases).

The duration of the folding process varies dramatically depending on the protein of interest. When studied outside the cell, some proteins require minutes or hours to fold primarily due to proline isomerization and often pass through several intermediate states. On the other hand, small single-domain proteins with lengths of up to a few hundred amino acids typically fold in a single step. Time scales of milliseconds are the norm.

Theoretical background to equilibrium unfolding

In its simplest form, equilibrium unfolding assumes that the molecule may belong to only two thermodynamic states, the folded state (typically denoted N for "native" state) and the unfolded state (typically denoted U). This "all-or-none" model of protein folding is believed to hold only for small, single structural domains of proteins; larger domains and multi-domain proteins often exhibit intermediate states. As usual in statistical mechanics, these states correspond to ensembles of molecular conformations, not just one conformation. The molecule may transition between the folded and unfolded states according to a simple kinetic model

$$N \rightleftharpoons U$$

Equation 1

with the rate constants k_f and k_u for the folding (U -> N) and unfolding (N ->U) reactions, respectively. The dimensionless equilibrium constant

$$K_{eq} = \frac{k_u}{k_f} = \frac{[U]_{eq}}{[N]_{eq}}$$

Equation 2

can be used to determine the conformational stability ΔG by

$$\Delta G = -RT \ln (K_{eq})$$

Equation 3

in which R is the gas constant and T the absolute temperature in Kelvin. Thus, ΔG is positive if the unfolded state is less stable compared to the native folded state.

The most direct what to measure the conformational stability ΔG of a molecule with two-states is to measure the kinetics of the folding and unfolding reactions. Because these often occur on the fast millisecond time scales these measurements are often inaccessible and special instrumentation, such as “stopped flow” or “continuous-flow is needed.

Chemical denaturation

In equilibrium unfolding experiment, the fraction of folded (N) and unfolded molecules (U) are measured in solution. To this end the solution conditions are gradually changed to favor either the native folded or the unfolded state. This can be achieved by gradually increasing the concentration of chemical denaturant. To commonly used and very well understood denaturants are urea and guanidinium hydrochloride.

At any given condition, the fraction of all populated species must sum to one and thus the conformational stability of the fraction of the native state can be derived from

$$pN = \frac{1}{1 + e^{\frac{-\Delta G}{RT}}}$$

Equation 4

and accordingly, the conformational stability of the unfolded fraction is derived from

$$pU = 1 - pN = \frac{e^{\frac{-\Delta G}{RT}}}{1 + e^{\frac{-\Delta G}{RT}}} = \frac{1}{1 + e^{\frac{\Delta G}{RT}}}$$

Equation 5

Protein stabilities are typically found to vary linearly with the denaturant concentration. A number of models have been proposed to explain this observation prominent among them being the denaturant binding model, solvent-exchange model and the Linear Extrapolation Model. All models assume that only two thermodynamic states are populated/de-populated upon denaturation. They could be extended to interpret more complicated reaction schemes.

The denaturant binding model assumes that there are specific but independent sites on the protein molecule (folded or unfolded) to which the denaturant binds with an effective (average) binding constant K. The equilibrium shifts towards the unfolded state at high denaturant concentrations as it has more binding sites for the denaturant relative to the folded state (Δn). In other words, the increased number of potential sites exposed in the unfolded state is seen as the reason for denaturation transitions. An elementary treatment results in the following functional form:

$$\Delta G = \Delta G_0 - RT\,\Delta n \ln\left(1 + K[D]\right)$$

Equation 6

ΔG_0 is the stability of the protein in water and [D] is the denaturant concentration. Thus the analysis of denaturation data with this model requires 7 parameters: ΔG_0, Δn, K , and the slopes and intercepts of the folded and unfolded state baselines.

The solvent exchange model invokes the idea of an equilibrium between the water molecules bound to independent sites on protein and the denaturant molecules in solution. It has the form:

$$\Delta G = \Delta G_0 - RT\,\Delta n \ln\left(1 + (K - 1)X_D\right.$$

Equation 7

in which K is the equilibrium constant for the exchange reaction and is the mole-fraction of the denaturant in solution. This model tries to answer the question of whether the denaturant molecules actually bind to the protein or they seem to be bound just because denaturants occupy about 20-30% of the total solution volume at high concentrations used in experiments, i.e. non-specific effects – and hence the term 'weak binding'. As in the denaturant-binding model, fitting to this model also requires 7 parameters. One common theme obtained from both these models is that the binding constants (in the molar scale) for urea and guanidinium hydrochloride (GdmCl) are small: ~0.2 M^{-1} for urea and 0.6 M^{-1} for GdmCl.

Intuitively, the difference in the number of binding sites between the folded and unfolded states is directly proportional to the differences in the accessible surface area. This forms the basis for the LEM which assumes a simple linear dependence of stability on the denaturant concentration. The resulting slope of the plot of stability versus the denaturant concentration is called the m-value. In pure mathematical terms, m-value is the derivative of the change in stabilization free energy upon the addition of denaturant. However, a strong correlation between the accessible surface area (ASA) exposed upon unfolding, i.e. difference in the ASA between the unfolded and folded state of the studied protein (dASA), and the m-value has been documented by Pace and co-workers.[3] In view of this observation, the m-values are typically interpreted as being proportional to the dASA. There is no physical basis for the LEM and it is purely empirical, though it is widely used in interpreting solvent-denaturation data. It has the general form:

$$\Delta G = m\,([D]_{\frac{1}{2}} - [D])$$

Equation 8

is called the "m-value"(> 0 for the above definition) and D1/2 represents the denaturant concentration at which 50% of the molecules are folded (the denaturation midpoint of the transition. In practice, the observed experimental data at different denaturant concentrations are fit to a two-state model with this functional form forΔG, together with linear baselines for the folded and unfolded states. Theandare two fitting parameters, along with four others for the linear baselines (slope and intercept for each line); in some cases, the slopes are assumed to be zero, giving four fitting parameters in total. The conformational stability ΔG can be calculated for any denaturant concentration (including the stability at zero denaturant) from the fitted parameters and

Absorbance spectroscopy

Absorption of electromagnetic radiation occurs when delocalized π-electrons are transferred from ground to an excited energy state. Various functional groups of proteins absorb UV light in the range from 150 to 300 nm. In the range from 180 to 240 nm the carbonyl group of the peptide bond absorbs predominantly, whereas from 250 to 300 nm mainly the aromatic ring system of phenylalanine, tyrosine and tryptophan absorb. The table below summarizes the absorbance properties of the aromatic amino acids and disulfide bonds.

Amino acid	λ_{max} (nm)	ε_{max} (M-1 cm-1)
Tryptophan	280	5700
Tyrosine	274	1400
Phenylalanine	257	200

Disulfide bond	250	300

Table 1 Comprehension of UV spectroscopic properties of the aromatic amino acids tryptophan, tyrosine, phenylalanine and disulfide bonds including the absorbance maxima and extinction coefficients

Spectroscopic properties depend on the polarity of the solvent. Wavelength shifts and changes in intensity can be observed when solvent polarity changes. Solvent polarity changes can give rise into conformational changes and changes in solvent accessibility of aromatic residues. The law of Lambert-Beer correlates the protein concentration with the measured absorbance.

$$A = \varepsilon\, c\, d$$

Equation 9 The law of Lambert-Beer, A: Absorbance, ε: molar extinction coefficient (M-1 x cm-1), c: molar protein concentration (M), d: layer thickness (cm).

Fluorescence spectroscopy

Absorption of photons can lead to transition of electrons into an excited state. When returning to ground state electrons dispose of their energy. The energy is either converted completely into vibration or partially converted into electromagnetic radiation, fluorescence. Electromagnetic radiation generated from relaxation of electrons from an excited state leads to emission of photons with smaller energy and in turn emitting at longer wavelength.

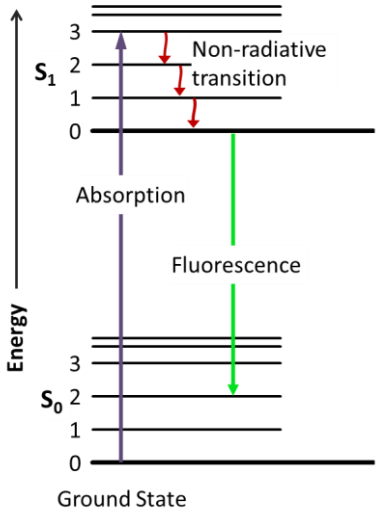

Figure 1 Jablonski diagram

Tryptophan, tyrosine, and phenylalanine exhibit intrinsic fluorescence due to their delocalized π-electron ring systems. Tryptophan is predominantly responsible for the fluorescence properties of a protein, as its extinction coefficient is larger than those of tyrosine and phenylalanine. The relative sensitivities for these three amino acids are: Trp : Tyr : Phe = 1100 : 200 : 8, respectively.

Trp fluorescence strongly depends on the solvent. The emission maximum of Trp in proteins ranges from 310 to 340 nm. The emission maximum of free tryptophan in hydrophobic environment is around 310 nm. In hydrophilic environment the maximum shifts towards longer wavelengths (> 350 nm).Thus, Trp fluorescence can be used to monitor the transition between conformational state of a protein, such as during denaturation/unfolding or folding, as buried Trp will show a wavelength shift when exposed to aqueous solvent upon unfolding. In addition, changes in fluorescence intensities may be observed. One explanation may be the loss of energy transfer from tyrosine, as the distance increases usually

when proteins unfold. A second explanation may be collision quenching of exposed tryptophan by solvent molecules. Tyrosine fluorescence shows a maximum at 303 nm, which does not change with solvent polarity though changes in intensity occur.

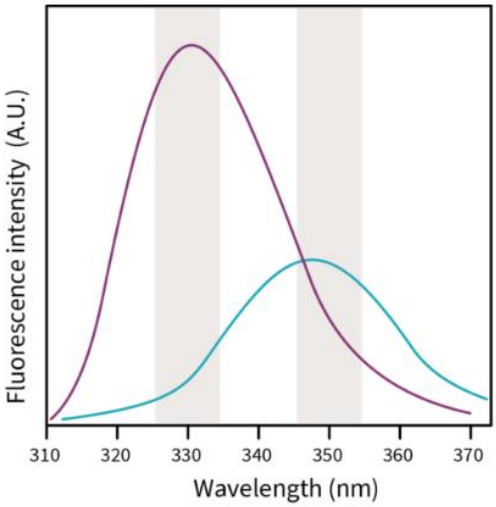

Figure 2 Fluorescence emission intensity spectra of a folded (purple) and unfolded (cyan) polypeptide

Fluorescence spectroscopy can be used to characterize the equilibrium unfolding of proteins by measuring the variation in the intensity of fluorescence emission or in the wavelength of maximal emission as functions of a denaturant value. The denaturant can be a chemical molecule (urea, guanidinium chloride), temperature, pH, pressure, etc.

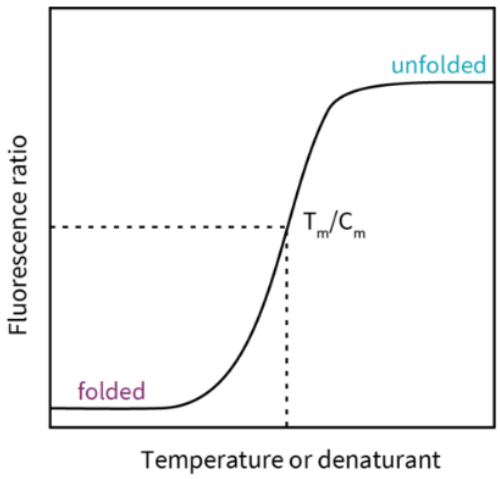

Figure 3 Schematic representation of a transition experiment in which the fluorescence ratio is plotted as the function of the temperature or denaturant concentration. The folded and unfolded baselines are depicted by "folded"and "unfolded", respectively. The transition midpoint is depicted by Tm/Cm indicating the temperature or denaturant concentration at which 50% of the molecules are folded and 50% are unfolded.

Determining the protein stability from chemical unfolding transitions

Preparation of 1x PBS buffer

- 0.5 mL 10X PBS
- 4.5 mL H2O
- Label 1X PBS

Preparation of 10 mL 8 M Guanidinium chloride solution

- Note: 8 M GdmCl make up about 50% of the total volume. Hence if you use your regular buffer to dissolve the amount of GdmCl needed, your buffer will be diluted by roughly 50%. Hence, one should prepare and use a xfold concentrated buffer to dissolve the amount of GdmCl needed.
- Weigh 7.6 g GdmCl (ultra-pure grade) into a 15 mL plastic vial
- Note: This amount of urea occupies roughly 50% of the final volume!
- Add 1 mL 10X PBS
- Slowly and carefully add H2O not exceeding 10 mL
- Place solution onto Vortex and mix to dissolve
- Note: The reaction is endotherm (gets cold). Mild heating / keeping it in your hand helps
- Add H2O to final 10 mL
- Label 8 M GdmCl
- Note: Usually you would now use a refractometer to determine the accurate molarity

Reagents for chemical unfolding of lysozyme

- 2.5 µM Lysozyme (100 µM Stock)
- 2.5 µM Bovine Serum Albumin (BSA) (100 µM Stock)
- Native Buffer: 1X PBS
- Unfolding Buffer: 8M GdmCl
- Equipment: Prometheus 48NT (NanoTemper), Room 212
- Standard capillaries
- Paper wipes

Experimental conditions for Prometheus NT48

- Time Control
- LED Excitation Power: 100%
- Isothermal: 25°C

Pipetting scheme of the chemical unfolding transition

NOTE: When pipetting, make sure that no extra liquid is on the outside of the pipette tip. Use a paper wipe to remove excess liquid. Extra material from the outside will compromise the measurements!

Sample #	[GdmCl] (M)	[Lysozyme] / [BSA] (µM)	**V Buffer (µL)**	**V GdmCl (µL)**	**V Lysozyme / BSA (µL)**
1	0	5	**95**	**0**	5
2	0.4	5	**90**	**5**	5
3	0.8	5	**85**	**10**	5
4	1.2	5	**80**	**15**	5
5	2	5	**70**	**25**	5
6	2.8	5	**65**	**35**	5
7	4	5	**45**	**50**	5
8	5.2	5	**30**	**65**	5
9	6	5	**20**	**75**	5
10	6.8	5	**10**	**85**	5
11	7.6	5	**0**	**95**	5

Table 2 Pipetting scheme for a chemical unfolding transition of Lysozyme using Urea as chemical denaturant.

- Incubate the reaction for 30 min at RT
- Record fluorescence emission intensity and fluorescence ratio according to the settings mentioned above

Analysis of the chemical-induced unfolding transition of Lysozyme and BSA

- Plot the fluorescence intensity at 330 nm as a function of the denaturant concentration
- Plot the fluorescence intensity at 350 nm as a function of the denaturant concentration
- Plot the fluorescence ratio as a function of the denaturant concentration
- Normalize all data to report the fraction unfolded
- Find the slope in the native and the unfolded baseline
- Determine the midpoint of the transition
- Determine the slope in the transition midpoint
- When available, analyze the transition at 25°C, 35°C, and 45°C
- Bonus: Using R, Matlab, Origin, Sigmaplot, Graphpad, etc, fit the data to the following model

$$F([denaturant]) = \frac{F_N - (F_N - F_U)}{1 + exp\frac{-\Delta G + m[denaturant]}{RT}}$$

Equation 10 Equation for non-linear chemical transition regression; F([denaturant]): regression as the function of the concentration of denaturant; F_N: signal for the natively folded protein, F_U: signal for the unfolded protein; m the cooperativity parameter

Determining protein stability from temperature-induced unfolding transitions

Like chemical denaturants, temperature also exerts an unfolding energy and thus can be used to unfold proteins. Similar to chemical-induced unfolding of proteins, in which the denaturant concentration is gradually increased to shift the fraction of molecules in the folded towards the unfolded state, temperature can also be used to test for the global stability of proteins. The native polypeptide is stabilized by a variety of interactions, including hydrophobic and electrostatic interactions. At some threshold temperature, the polypeptide becomes unstable and adopts the unfolded state and as the temperature gradually increases, the fraction of unfolded molecules increases. The cooperative behavior of protein folding is reflected by the non-linear change in the fraction of folded molecules and allows determining the melting temperature for the protein under investigation.

Assuming a defined folding path, such as a classical two-state folding system (N-U), dG, dH and dS can be determined, provided the heat capacity of the protein is known (calculated or experimentally determined). Temperature-induced unfolding often coincides with irreversible aggregation of the unfolded polypeptide. In such case, the system is no longer in equilibrium and a proper thermodynamic analysis is hampered.

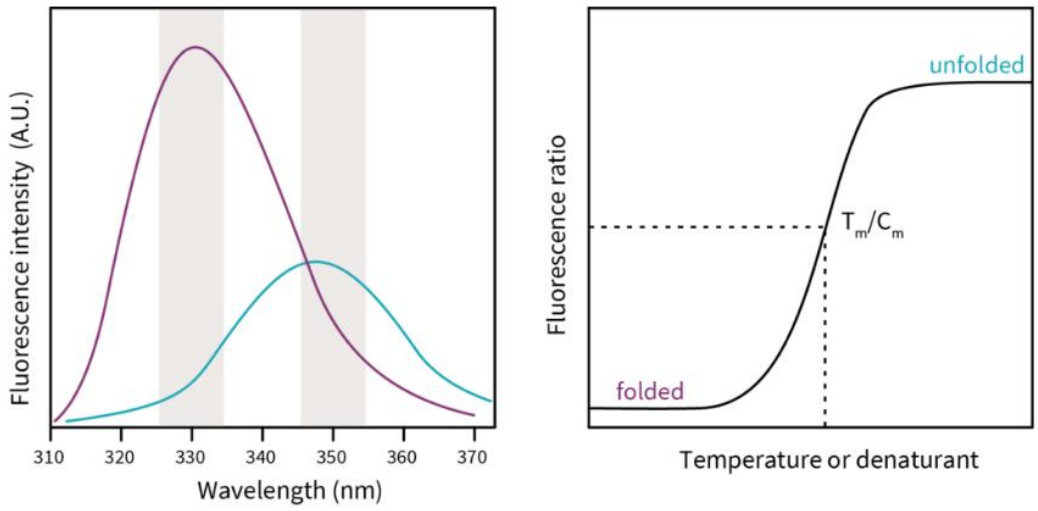

Figure 4

Reagents for temperature-induced unfolding of Lysozyme and BSA

- Lysozyme 100 µM stock
- BSA 100 µM stock solution
- Native Buffer: 1X PBS
- Reaction volume: 40 µL
- Prometheus Panta (NanoTemper)
- Standard capillaries

Experimental conditions for Prometheus Panta

- Temperature increment 2°C / min
- Start: 20°C, End: 95°C
- DLS during unfolding: ON

Sample setup for temperature-induced unfolding transition

Capillary	[Lysozyme] (µM)	**V Buffer (µL)**	**V Lysozyme (µL)**	[BSA] (µM)	**V Buffer (µL)**	**V BSA (µL)**
1	2.5	**39**	**1**			
2	5	**38**	**2**			
3	10	**36**	**4**			
4				2.5	**39**	**1**
5				5	**38**	**2**
6				10	**36**	**4**

Table 3 Pipetting scheme for a temperature-induced unfolding transition of Lysozyme and BSA using

- Load capillaries with respective protein solution
- Place capillaries into Prometheus (NanoTemper)
- Set Start temperature to 15°C
- Set End temperature to 95°C
- Set temperature ramp to 2°C/min
- DLS during melting: OFF

Analysis of temperature transition

- Plot the fluorescence at 330 nm as a function of the temperature
- Plot the fluorescence at 350 nm as a function of the temperature
- Plot the fluorescence ratio F350/F330 nm as a function of the temperature
- For all samples find the transition midpoint
- Inspect and analyze the light scattering signal

Lysozyme aggregation assay

The reduction of cysteines of Lysozyme causes a destabilization of the folded polypeptide that leads to unfolding. This unfolding is coupled to non-specific, irreversible association of polypeptide chains, called protein aggregation. Often protein aggregates grow to large sizes. Large particles, such as protein aggregates scatter light and accordingly the aggregation can be followed analytically using a UV spectrophotometer or fluorometer.

In this part of the course we will use lysozyme as a model to monitor its aggregation and the effect of the small heat shock protein HspB1 on the aggregation of lysozyme. As small heat shock proteins are considered as molecular chaperones that inhibit non-productive side reactions, such as aggregation, we expect to find that the formation of large light scattering particles decreases in the presence of the small heat shock protein HspB1.

Reagents for Lysozyme aggregation assay

- Lysozyme 100 µM stock
- Native Buffer: 1x PBS
- Equipment: Tecan M200Pro Plate Reader
- Reaction volume: 100 µL
- TCEP stock solution (500 mM)
- 96-well plate, Type
- M200Pro Plate Reader (Tecan), Room 210

Experimental conditions for Tecan M200Pro Plate Reader

- Temperature: 37°C
- Absorbance: 400 nm
- Shaking: X

Setting up a Lysozyme aggregation assay to test for Hsp27 activity

Sample	[Lysozyme] (µM)	**V Lysozyme (µL)**	[Hsp27] (µM)	**V Hsp27 (µL)**	V TCEP (µL)	**V Buffer (µL)**
1	10	**10**	0	**0**	1	**89**
2	10	**10**	5	**2.5**	1	**84**
3	10	**10**	10	**5**	1	**79**
4	10	**10**	20	**10**	1	**74**
5	10	**10**	0	**0**	0	**90**
6	0	**0**	20	**10**	1	**89**

Table 4 Pipetting scheme for a Lysozyme aggregation assay to test for the activity of the small heat shock protein Hsp27

Analysis of effect of HspB1 on the chemically induced aggregation of Lysozyme

- Plot the light scattering signal (Absorbance @ 400 nm) as a function of time
- When necessary, normalize the individual data to start at 0
- Determine the apparent half time that is needed to aggregate lysozyme w/o and w/ HspB1
- Determine the apparent inhibition of lysozyme aggregation from the plateau values in the presence of different concentration of HspB1

Michaelis-Menten kinetics

Enzymes are catalytically active proteins. They exhibit the ability to speed up the turnover of a substrate into a product, some of which can accelerate the process 10^8 to 10^{12}fold. To this this end, the enzyme binds to the substate to form a enzyme-substrate complex, which subsequently turns the substrate into the product. The general reaction scheme is depicted by

$$E + S \underset{k_r}{\overset{k_f}{\leftrightharpoons}} ES \xrightarrow{k_{cat}} E + P$$

Equation 11

where k_f and k_r are the rate constants for binding and dissociation of the substrate (S) to the enzyme (E), respectively to form the enzyme-substrate complex (ES) and k_{cat} is the rate constant of catalysis at which the substrate (S) is turned over to product (P). Note: Enzymes are no constituent of the product.

In biochemistry, Michaelis–Menten kinetics (Leonor Michaelis and Maud Menten) is one of the best-known models of enzyme kinetics. The model takes the form of an equation describing the rate of enzymatic reactions, by relating reaction rate v, the formation of the product, [P] from the substrate [S].

$$v = \frac{d[P]}{dt} = \frac{V_{\max}[S]}{K_M + [S]}$$

Equation 12

where, V_{max} is the maximum rate by which the system can turn over the substrate into product at saturation substrate concentrations and K_M is the Michaelis-Menten constant, the numerical value of the substrate concentration at which the reaction rate is half ($V_{1/2}$).

Lactate dehydrogenase

Lactate dehydrogenase is an important enzyme of the metabolic pathway of glucose. It catalyzes the turnover of pyruvate to lactate and requires the cofactor NADH. This reaction is particularly important during muscle work. Produced lactate is transported from the muscle with the blood stream to the liver, where the same enzyme, LDH, catalyzes the reverse reaction to rebuilt pyruvate (subsequently glucose through gluconeogenesis) from lactate and NAD^+. This cycle of glucose and lactate between the liver and the muscle is called the Cori-cycle and warrants muscle work even during anaerobic conditions.

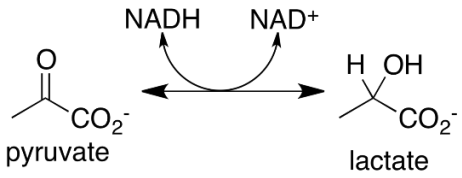

Figure 5 Reaction catalyzed by lactate dehydrogenase

Setup LDH enzyme assay

- Native Buffer: 1X PBS
- LDH stock solution
- 50 mM NADH in PBS
- 50 mM Pyruvate in PBS

- Equipment: NP80 (Implen), Room 201
- Reaction volume: 500 µL

Experimental conditions for Implen NP80

  - Cuvette, disposable micro cuvette 8.5 mm center hight
  - Temperature: 37°C or RT, to be determined
  - Absorbance: 340 nm
  - Kinetics module, Duration: 1 min, Interval: 5 s

Preparation of 100 mM Pyruvate stock solution

- Weight in 0.22 g Pyruvate
- Dissolve in 25 mL PBS
- Sterile filter with 0.22 µm
- Label: 100 mM Pyruvate in PBS
- Make 100 µL aliquots

Preparation of 50 mM NADH stock solution

- Weight in 0.17 g NADH
- Dissolve in 5 mL PBS
- Sterile filter with 0.22 µm
- Label: 50 mM NADH in PBS
- Make 50 µL aliquots

Reaction Mix (7.5 mL)

PBS (1X)		
LDH (X U / mL)	2 U / mL	X µL
NADH (50 mM Stock)	250 µM	37.5 µL
Total		7500

- 7500 µL Total
- PBS
- X µL LDH (2 U / ml)
- 250 µM NADH

Analysis of Enzyme kinetics

- Find the initial rate of catalysis v0 from the individual reactions
- Calculate the change in NADH concentration for each substrate concentration
- Plot the apparent reaction rate as a function of substrate concentration
  - Extinction coefficient of NADH ~ 6200 1/M 1/cm
- Determine v_{max} and K_M either manually or by regression of the data
- Plot the data as a Lineweaver-Burk plot.

2X Pyruvate Dilution Series								
Solution No	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
[Pyruvate] (mM)	100	50,00	25,00	12,50	6,25	3,13	1,56	0,78	
[Pyruvate] in reaction (mM)		5,000	2,500	1,250	0,625	0,313	0,156	0,078	
Volume Pyruvate Solution (µL)		100 µL No 0	100 µL No 1	100 µL No 2	100 µL No 3	100 µL No 4	100 µL No 5	100 µL No 6	
		+	+	+	+	+	+	+	
Volume PBS (µL)		100 µL PBS	100 µL PBS	100 µL PBS	100 µL PBS	100 µL PBS	100 µL PBS	100 µL PBS	

2X Pyruvate Dilution Series							
Solution No	**7**	8	9	10	11	12	13
[Pyruvate] (mM)		0,39	0,20	0,10	0,05	0,02	0,01
[Pyruvate] in reaction (mM)		0,039	0,020	0,010	0,005	0,002	0,001
Volume Pyruvate Solution (µL)		100 µL No 7	100 µL No 8	100 µL No 9	100 µL No 10	100 µL No 11	100 µL No 12
		+	+	+	+	+	+
Volume PBS (µL)		100 µL PBS	100 µL PBS	100 µL PBS	100 µL PBS	100 µL PBS	100 µL PBS