Final report of the project K-84090 (The oscillation reaction of hydrogenase)

Aims and scope

The aim of this project was twofold. First we planned to characterize the autocatalytic reaction of the hydrogenase enzyme. The previous experiments were planned to extend in order to vary the temperature, pH and hydrogen gas concentration in the reaction and measure characteristic parameters of the reaction like front velocity, enzyme activity, saturation values, etc.

Measuring the temperature dependence of the rate constants, in a given model the activation energy and other thermodynamic parameters can be derived.

Although we already knew that the autocatalyst form of the enzyme is directly interacting with the oxidized electron acceptor, we do not know the other enzyme form that is catalyzed by the autocatalyst. Intensive model simulations and calculations, as well as experimental work were planned in order to identify the autocatalyst form of the enzyme.

The second aim was to optimize the conditions for oscillatory behavior and characterize it for the hydrogenase enzyme system and explain the unusual behavior (apparently the final product of the reaction is oscillating) of this oscillatory reaction.

Results

Technical achievements

1. A new reaction chamber (Temperature Controlled Thin Layer Reaction Chamber, TC-TLRC) was designed and built. The concentration of the reaction product/substrate (reduced benzyl or methyl viologen) was followed by measuring the optical density of the reaction mixture. We used the previously described thin-layer reaction chamber with the addition that a prism was applied at a fixed position on the top of the chamber. The light collected by the prism was introduced through an optical fiber into an Ocean Optics USB-2000+ spectrometer. The transmission spectrum of the light penetrating through the sample was recorded in the interval 200-1100 nm, using the SpectraSuite software. The measurements were continued until the measured transmission values apparently no longer changed in the visible spectral range (the UV range was neglected since the glass parts of the system absorbed the UV part of the spectrum). Later, in order to increase the light for detection, a Fresnel lens was applied on the top of the TLRC this way the optical fiber collected the penetrating light from wider area of the reaction chamber.
2. This new design also allowed controlling the temperature of the reaction. The bottom part of the previous design was extended with another compartment through which temperature-controlled water flowed continuously. The water cooled the bottom part of the anaerobic chamber in which the thin-layer sample holder with the reaction mixture was located. The temperature was monitored with an Fe-constantan

thermocouple inserted into the bottom wall of the anaerobic chamber. The bottom wall, made from Plexiglass, was kept as thin as possible in order to facilitate the best thermal contact between the water and the thin-layer sample holder. The temperature of the reaction mixture was calibrated before the experiment with another thermocouple immersed directly into the reaction mixture. During the measurements, this thermocouple was removed in order to maintain anaerobicity, and the monitoring thermocouple together with the calibration curve was used to determine the actual temperature. The error in the temperature determination was estimated to be ±2 ˚C.

3. The gas supply was also modified. With the new design we were able to control the H_2 content of the gas flushing through the headspace of the reaction chamber.
4. With the help of all these changes it became possible to run the reaction back and forth several times (cycling). The possible number of cycles was limited because of the evaporation of the reaction medium due to the intensive flushing with gases. The evaporation of the water from the sample and the precipitation of the vapour in the sample holder depended strongly on the temperature differences between the sample, the sample holder and the gas. In order to minimize the drying, the gas was bubbled through a water bubbler to saturate it with vapour and it was cooled (warmed) before entering the reaction chamber in the water bath that maintained the constant temperature of the sample. Consequently, when it entered the sample chamber, the gas was at approximately the same temperature as the sample in the sample holder. If this had not been done, the sample would have dried out in a short time.

   This arrangement allowed the first time to measure precisely both the H_2 oxidation and proton reduction activities of exactly the same enzyme sample. By repeating the H_2 oxidation/proton reduction cycles several times, different forms of the enzyme (ready/unready, active) could be studied and the reaction parameters for different enzyme forms could be determined.
5. We have developed a MATLAB program in order to evaluate the cycling H_2 oxidation and proton reduction experiments. All the parameters (lag phase, activity, (apparent) saturation values are derived.

Scientific achievements

Activation of hydrogenase

If the traditional experimental model is correct, there is only one way for electrons to activate the Ni-A and Ni-B forms under gaseous H2 with no electron acceptor present. A very low number of enzyme molecules are present not in the Ni-A or Ni-B forms, but in an "active" form, and these enzyme molecules may bind and split H_2 to produce electrons. These electrons are located in the [FeS] clusters. If 2 hydrogenase molecules are in contact, one with its distal cluster reduced is able to transfer an electron to the not reduced distal cluster of another hydrogenase molecule, which is in Ni-A or in Ni-B form. This electron passes along the [FeS] cluster chain to reach the [NiFe] cluster and reduce it to a silent form. In this way, a new enzyme molecule is produced which is able to participate in H_2 splitting. This is an autocatalytic process, the interaction of the two enzyme molecules comprising an autocatalytic reaction step. It is most likely this autocatalytic step that causes the long lag phase of the reaction during H_2-oxidation measurement, and the appearance of growing spots in thin layer experiments. When consecutive H_2-oxidation and proton-reduction experiments are performed on the same sample by changing the gas atmosphere above the reaction volume, these characteristics are present only in the first H_2 oxidizing step. None of the

subsequent H_2 oxidizing steps exhibit the long lag phase and no spots can be observed at the beginning of the reaction. Consequently, full activation of the enzyme eliminates this autocatalytic reaction step.

The catalytic cycle of hydrogenase

1. **Contradiction in the traditional enzyme cycle models**
   We have developed a full hydrogenase catalytic model based on the structure of hydrogenase (4 different redox centers) and all the theoretically possible steps of H_2 splitting (five or four consecutive steps, depending on the reaction order). The construction resulted several parallel models (depending on the concrete order of reaction steps in H_2 splitting) with 40 enzyme intermediers. The differential equation of the model was also constructed, although the exact placement of the autocatalytic step is not yet determined.

   When a traditional model (with no autocatalytic step in the reaction cycle) contains heterolytic H_2 splitting and no electron acceptor is present in the reaction (imitating incubation of hydrogenase under hydrogen), the graph depicting the enzyme reaction cycle splits into two parts (two separate graphs). One part of the graph contains enzyme intermediers with only even number of electrons in the whole enzyme, while the other part of the graph contains only odd number of electrons. Consequently the final intermediers in the two graphs have different magnetic properties, one is diamagnetic, and the other one is paramagnetic. This is in direct contradiction with the experiment. When incubating the hydrogenase under hydrogen the result is always a diamagnetic enzyme form. Consequently the traditional model cannot be valid.

2. **There is an autocatalytic step in the enzyme cycle of hydrogenase**
   We postulated a possible autocatalytic step in the hydrogenase reaction cycle. Experimentally, this is supported by the fact that the Ni-R form is exclusively diamagnetic. It has emerged that the final steady state of the H_2-oxidation reaction depends on the enzyme concentration. In other words, the final “blueness” of the sample is enzyme concentration dependent. If more enzyme is added to the same amount of oxidized electron acceptor (i.e. benzyl or methyl viologen), when the reaction stops it will be “bluer”. This is impossible for a “normal” enzyme cycle. A catalyst (enzyme) can never affect the extent of the reaction it catalyzes; that would be in contradiction with the second law of thermodynamics.

   If consecutive H_2-oxidation and proton-reduction experiments are carried out on the same sample by changing the gas atmosphere above the reaction volume, the enzyme concentration dependence of the apparent saturation level remains true for all subsequent H_2 oxidation steps in the experiment, and not merely for the first one. This means that “activation” does not affect this behavior. There might be a difference between the final levels depending on the activation, but the dependence does exist.

   The only explanation appears to be that the enzyme is withdrawn from the reaction at a certain point and the reaction cycle is stopped. This can be achieved if an autocatalytic step is assumed inside the enzyme cycle. We have not discovered any alternative acceptable explanation. This would be a second autocatalytic step in the hydrogenase reaction, located inside the enzyme cycle.

   The interacting partners should also be different enzyme forms. If the autocatalyst

(this enzyme form should be later in the enzyme cycle) disappears, the concentration will drop below 1 molecule/reaction volume, the enzyme cycle will then stop and the concentration of the reduced electron acceptor will be frozen at this point. This point, however, depends on the amount of enzyme present. If there is more enzyme, the autocatalyst will disappear later and more reduced electron acceptor will be produced. If the reaction direction is changed by altering the gas atmosphere, the opposite reaction can easily occur since the autocatalyst and the other enzyme form change their roles: the other interacting enzyme form becomes the autocatalyst and, with plenty of the autocatalyst, the reaction can proceed. If the atmosphere is changed back to gaseous H_2 the whole procedure will be repeated.

The other interacting enzyme form (the enzyme substrate for the autocatalyst) can be enriched if the reaction is allowed to proceed until it stops. At that moment the entire enzyme should be in this form. Measurement of this enzyme form with a suitable spectroscopic method will shed light on the nature of this interacting partner. Such experiments are currently in progress.

3. **The autocatalytic step can also be evinced in the proton reduction direction**
When subsequent H_2 oxidation and proton reduction experiments are performed, a definite lag phase can be observed in proton reduction. It is present in every cycle, and does not depend on the active-inactive state of the enzyme.

Oscillating hydrogenase reaction

Through utilization of the autocatalytic nature and the multiple roles of protons in H_2 uptake and production, together with limitation of the substrate (gaseous H_2) supply, oscillations can be initiated in the reaction chamber. Oscillatory behavior can be induced if the reaction mixture is not buffered and the initial gaseous H_2 concentration in the atmosphere is set to 5-10%. The oscillation can be observed as a periodic color change of the product (benzyl viologen or methyl viologen) as it changes its oxidation state. The oscillation can also be followed by measuring the optical density. The oscillation has a long period, which increases considerably further as time passes. Stirring the reaction mixture affected neither the existence of oscillation nor the oscillatory characteristics. Without stirring, however, convection, too, plays a role in the spatial distribution of the reaction.

When the reaction is carried out in a thin-layer reaction chamber, it can be demonstrated that the reaction oscillates both in space and in time. The reaction starts similarly to the buffered reaction, blue spots of the reduced electron acceptor (benzyl viologen) appear at different positions in the originally colorless reaction volume and the spots expand with constant velocity. A colorless spot then appears in the centre of the blue spots, indicating the occurrence of the reverse reaction in the middle of the reaction circle.

The further evolution of the process in thin layer is apparently stochastic. The reaction volume sometimes oscillates as a whole. Moving fronts of reduced and oxidized benzyl viologen are seen at times, wandering around randomly the reaction surface. Propellers may also be generated, which rotate along the side of the reaction chamber.

Unfortunately we were yet unable to build a mathematical model that describes this phenomenon. Several attempts has been made to approximate this behaviour. The problem is, that apparently the end product is oscillating which seems to be impossible for a simple chemical reaction. The reaction should be very far from the equilibrium state. We still making efforts to make a correct and feasible model to calculate the observed oscillations in the hydrogenase reaction.

Temperature dependence of hydrogenase activity

We suggested a conformation change in the hydrogenase catalytic cycle, when the autocatalytic interaction between two different enzyme intermediers happens. In order to follow this hypothetic conformational change a temperature-dependent kinetic and light-scattering study was performed. Three different temperature regions were identified depending on the kinetic and light scattering behavior of the enzyme: ~8-14 ºC, 14-28 ºC and above 30 ºC. Above 30 ºC, the reaction-diffusion fronts disappeared, and the obvious autocatalytic behavior of hydrogenase could not be observed. The saturation level for the product (benzyl viologen) was the highest in this region. Below 30 ºC, the reaction-diffusion fronts were very pronounced; the saturation level was slightly lower than above 30 ºC. Below ~15 ºC, the activation energy for the hydrogenase reaction increased by a factor of two, and the saturation level dropped significantly.

Dynamic light-scattering experiments supported the differences in protein behavior in the different temperature ranges. Changes were observed in Z-average and the average protein hydrodynamic diameter in the border regions of the temperature ranges, indicating rearrangements in the protein structure.

Exactly what happens with the protein at these temperatures is not yet known; the hypothetic slight conformational changes (the tertiary or quaternary structure might change) or changes in the conformational movement of the hydrogenase are, however, supported by the experiments.

Hydrogen concentration dependence of hydrogenase activity

In these experiments the second substrate of the reaction, the H_2 concentration was changed. The activity and saturation values were calculated for all reaction cycles. There was a marked decrease in all activities as H_2 concentration decreases, till around the 1% H_2 concentration in the atmosphere. Below this concentration the activity did not change. Unfortunately – as it turned out – this was due to a gas mixer fault and does not represent a real effect.

A marked difference between the first and the following two hydrogen oxidizing cycles was also present. If the H_2 concentration increased, the difference between the first cycle and the other two cycles also increased.

Saturation values showed the same tendency as the activity. With decreasing H_2 concentration the apparent saturation decreased. The artefact due to the gas mixer fault is also observable.

Effect of Hofmeister salts on the activity of hydrogenase

Hydrogen oxidation and proton reduction activities were measured in the presence of different Hofmeister salts with the use of benzyl viologen as electron acceptor/donor. Kozmotropic and neutral salts had practically no effect on the activity of hydrogenase, whereas chaotropic salts gave rise to dramatic effect. In the presence of chaotropes H_2-oxidation (benzyl viologen reduction) took place readily, but proton reduction (benzyl viologen oxidation) was blocked.

The concentration, at which the blocking appeared depended on the nature of the Hofmeister salt. As a rule, it was observed that a salt that was more chaotropic caused the block at a lower concentrations. For $NaNO_3$, the transition was at around 6-8 mM, and at a concentration of 20 mM the proton reduction was completely blocked. For NaSCN, the transition took place below 1 mM, and at 1 mM the proton reduction was already blocked completely. It was very unusual that the concentration at which the inhibition appeared was rather low. The effects of Hofmeister salts on the enzyme activity are customarily seen at around 100-200 mM, whereas the concentration-dependent transition in our case occurred at a

salt concentration of around 1-20 mM, i.e. very close to the benzyl viologen concentration. It rather suggested the effect of Hofmeister salt on benzyl viologen than in protein.

Since viologens have found widespread applications in many fields as electron transfer mediators or chromophores, therefore we have extended our study to investigate the redox properties of benzyl viologen in the presence of Hofmeister salts and found a very interesting effect, not anticipated when writing the grant application. These results are a side effect of our study.

Unusual redox properties of dibenzyl viologen in the presence of Hofmeister salts

In photometric titrations, we were apparently unable even to reduce the benzyl viologen if sufficient chaotropic salt was present (the benzyl viologen did not change color). The dependence of OD_{600} at the saturation potential (~ -600 mV) on the salt concentration was very similar to that observed in the case of enzymes.

CV measurements corroborated these results. In the case of chaotropic salts, the redox potential shifted in the positive direction and a different redox couple was observed, depending on the chaotropic behavior of the salt. The redox potential of this new redox couple varied with increasing chaotropic salt concentration. The concentration-dependent transition was observed at the same concentration as in the previous experiments.

In contrast with the electrochemical experiments, on use of the enzyme it was possible to reduce the benzyl viologen in a "normal" manner (the benzyl viologen became blue) even at very high concentrations of the chaotropic salts; only the proton reduction step (benzyl viologen oxidation) was blocked. It should also be noted that, when reduced benzyl viologen was oxidized by air, the enzyme was able to re-reduce the viologen dye repeatedly. This is a marked difference between the electrode and enzyme experiments.

We have to note that in the case of methyl viologen none of the effects present in the case of benzyl viologen could be observed.

We cannot explain yet what exactly happens in the case of chaotropic salts and benzyl viologen. It is clear that no chemical reaction occurs because the transition concentration depends strictly on the chaotropic strength of the anion in the Hofmeister series. Our preliminary FTIR measurements show that some (as yet unassigned) bands shift in the presence of chaotropic salts, but not if kozmotropic or neutral salts are present. NMR experiments, however, did not present any change in the structure of benzyl viologen.

Molecular dynamics calculation also did not show any difference between the two viologens.

We cannot exclude the role of the surface in our experiments, since the reaction takes place on the electrode surface (even the enzyme itself can be treated as a surface for the reaction). It is noteworthy, however, that the effect is seen at much lower salt concentrations than a "normal" Hofmeister effect on enzyme activity.

Binding of viologen molecules on the surface of hydrogenase

In cooperation with Csaba Hetényi (Molecular Biophysics Group, HAS-ELTE) we have calculated the binding sites of the benzyl- and methyl viologen molecules to the surface of the hydrogenases. Two hydrogenase molecules were taken into account (*Allochromatium vinosum* PDB 3MYR and *Thiocapsa roseopersicina*, calculated). Recently the electron transport pathways are calculated from/to the iron-sulphur clusters and the FeNi center in the hydrogenase.

Autocatalytic reaction in other hydrogenases

In cooperation with Wolfgang Lubitz from Max Planck Institute für Chemische Energiekonversion we have investigated the kinetic properties of a hydrogenase from

Desulfovibrio gigas using our TC-TLRC. This is an important enzyme, being the most investigated hydrogenase and it is accepted as a prototype of NiFe hydrogenases. All the characteristic properties of an autocatalytic reaction what we have found using *Thiocapsa roseopersicina* hydrogenase were present in the case of *D.gigas* hydrogenase.

Another hydrogenase was also investigated although with less extent. It was an oxygen tolerant hydrogenase of *Ralstonia eutropha*. This work was done in cooperation with Oliver Lenz from Technische Iniversitet Berlin. This hydrogenase was routinely assayed by methylene blue which did not allow us the observation of reaction-diffusion fronts (it has a dark blue color in oxidized state). We have detected, however, the enzyme concentration dependent lag phase which is also characteristic for autocatalytic reactions.

Educational achievements

Two PhD thesis will be prepared. One of them (Sarolta Bankó) is already written. She has to defend when her second publication will appear. The other one (Zsuzsanna Janovics) has not yet been written her thesis because she left for mathernity leave.

Publications

Three publications has already been published in peer reviewed journals as seen in the publication list. We have other four manuscripts ready, three of them has already been in different journals for publication (one about the temperature dependence of the hydrogenase activity, another one about the temporary dimer formation of the hydrogenase molecules in the reaction cycle, and the third about the effect of Hofmeister salt on the electrochemistry of benzyl viologen). Unfortunately they were rejected. The fourth manuscript is a new one, and it is description and analysis of the hydrogenase activity in the newly developed thin layer reaction chamber.

We hope that all four would be published in near future, therefore we would like to include them into the publication list when it would be appropriate.

Two more manuscripts are in preparation. They will describe the autocatalytic behavior measured in the case of other hydrogenases (hydrogenase from *Desulfovibrio gigas* in cooperation with Wolfgang Lubitz from Max Planck Institute für Chemische Energiekonversion, and the oxygen tolerant hydrogenase from *Ralstonia eutropha* in cooperation with Oliver Lenz, Technische Iniversitet Berlin).

We have several conference presentations, some of them has been published in conference proceedings. They are almost all listed in the publication list.