ENZYMATIC UPGRADING OF BIOGAS

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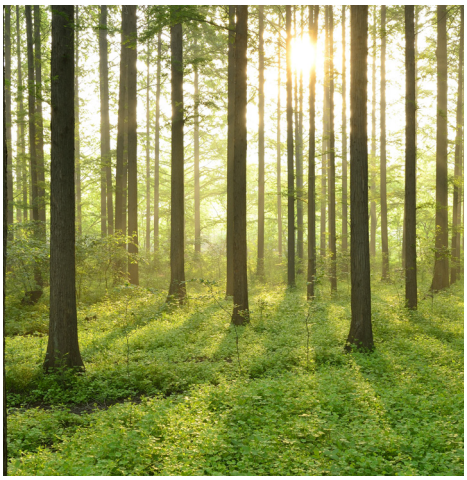
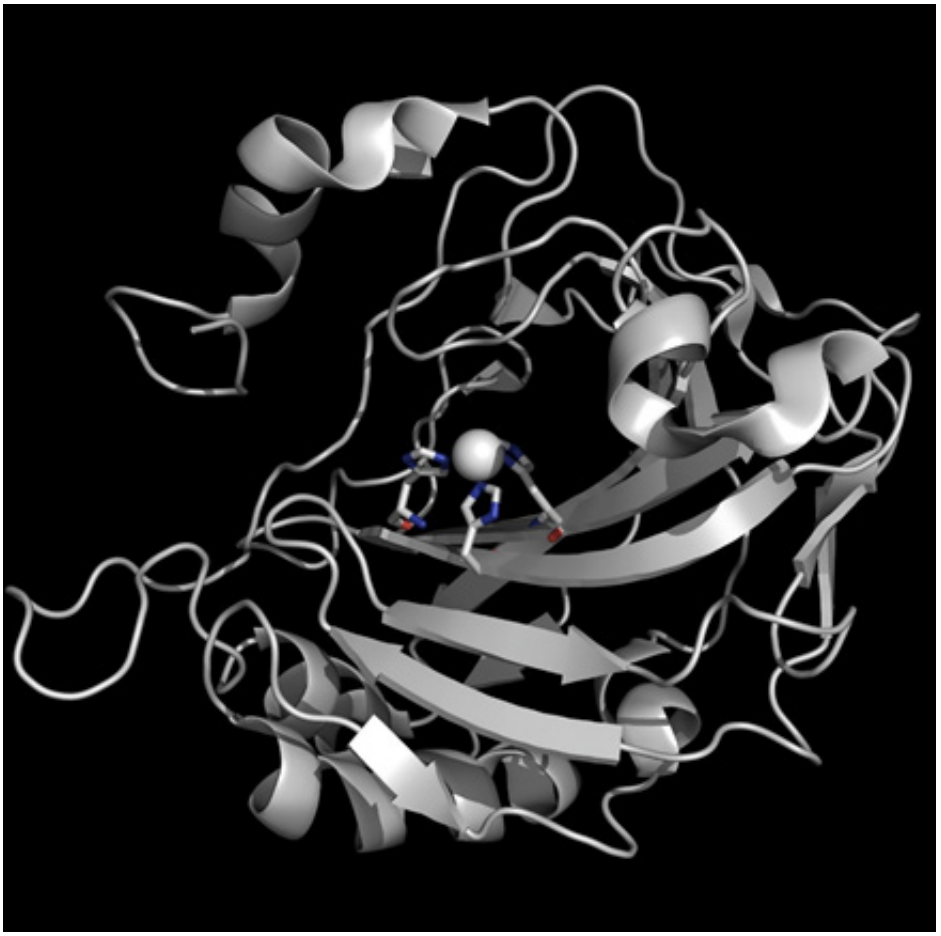


ENERGY GASES AND LIQUID FUELS



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Enzymatic upgrading of biogas

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Authors' foreword

The following findings and results have their origin in the project "Enzymatic upgrading of biogas". The project was originally intended to run for two years and cover literature studies on the current state of enzyme based carbon capture & sequestration (CCS) in order to design, test and build an enzyme based CO_2 separation process, but for the application of biogas upgrading. The project was initiated based on the fact that involved stake holders had access to an engineered enzyme (InZymes Biotech AB) that was thought to be a promising candidate enzyme for a biogas upgrading implementation, if the process it was incorporated into (Purac Puregas AB) would provide the correct conditions for the enzyme to remain active over the necessary period of time to generate an economically viable process.

Unfortunately, time plans and budget regarding the laboratory setup of the process could not be sustained and the project was prematurely terminated after one year for the benefit of other projects within the framework program of the Swedish Energy Agency, "Co-operational program in Energygas technology". Thus practical implementation in laboratory tests of the process had to be abandoned. Nevertheless, very much was learned and many insights were gained which we try to convey in this report.

The project was conducted between December 2013 and January 2015. The project was coordinated from Linköping University, Dept. of Physics, Chemistry and Biology, and different parts of the project was carried out at Linköping University, Purac Puregas AB in Kalmar and Medicago AB in Uppsala. The report was authored by Martin Karlsson and Patrik Nygren. Four reference groups meetings was held at Linköping University during the project. All meetings included the presence of the respective project leaders:

- Martin Karlsson, InZymes Biotech AB/Linköping University
- Lars-Evert Karlsson, Purac Puregas AB
- Jan Henriksson Medicago AB

At different times also reference persons and technicians including: Uno Carlsson, Linköping University; Bengt-Harald Jonsson, Linköping University; Anton Linde, Medicago AB; Roger Nilsson, Swedish Biogas International AB; Patrik Nygren, Linköping University/InZymes Biotech AB; Tobias Persson, Swedish gas technology centre (SGC); Max Strandberg, Purac Puregas AB.

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Martin Karlsson, Linköping University, Aug 2015

Sammanfattning

Det högsta förädlingsvärdet av biogas är i form av uppgraderad biogas för fordon eller inmatning på gasnät där biogasens innehåll av metan har avskilts från koldioxiden. Etablerade metoder för uppgradering baseras på höga tryck eller temperaturer och är därmed mycket energiintensiva. Höga kostnader för uppgradering har därför stor inverkan på biogasproduktionens ekonomi. Vidare så är etablerade teknikers skalbarhet låg och ofta inte anpassade för småskalig biogasproduktion. Båda dessa omständigheter lägger hinder för biogasproduktionens utveckling och expansion.

Forskning och satsningar på processutveckling för koldioxidinfångning och lagring (Carbon Capture and Sequestering, CCS) har varit mycket mer omfattande. CSS hanterar till stora delar samma problem som de i biogasuppgradering, d.v.s. att fånga koldioxiden ur gasblandningar, men med än större incitament att fungera energieffektivt då CCS inte ger något mervärde vid sidan av miljöeffekterna av att fånga och lagra koldioxid. All energiåtgång vid CCS är alltså ur ett kostnadsperspektiv rent parasitisk energiåtgång. Därför har också stora resurser och ansträngningar lagts på forskning för att hitta alternativa lösningar för energieffektiv CCS med lågt parasitiskt energibehov.

Några av de mest effektiva metoderna för CCS utnyttjar aminer som lösningsmedel för CO_2, av vilka några dock är ”för reaktiva” och bildar starka bindningar med CO_2. Som konsekvens krävs därför höga temperaturer och mycket energi för att kontinuerligt regenerera aminlösningsmedlet. Mindre reaktiva lösningsmedel finns, men den lägre reaktiviteten, med dess motsvarande lägre reaktionshastighet, innebär att de inte är tillämpliga för CCS då absorptionstorn skulle behöva vara mycket stora/höga och flödeshastigheter skulle behöva vara låga för att utnyttja den koldioxidbindande kapaciteten hos dessa aminlösningsmedel. För att kringgå den låga reaktions-hastigheten hos lågenergilösningsmedel har det föreslagits att använda enzymer (karboanhydraser) för att öka reaktionshastigheten. För att en enzymbaserad CO_2-infångande process skall fungera krävs dock att hänsyn tas till alla ingående delar, innefattande lösningsmedel, process och enzym, och att dessa kan anpassas till varandras begränsningar. Vi har därför i detta projekt studerat litteratur och patent som avser forskning i alla dessa delar med avsikten att designa och bygga en energieffektiv enzymbaserad biogasuppgraderingsprocess i laboratorieskala.

Med avseende på processen så finns flera alternativ presenterade i litteraturen. Alla dessa använder enzymer immobiliserade i antingen absorptionsfasen, alternativt i både absorptions- och desorptionsfasen. Detta behov av att immobilisera enzymet kan sannolikt orsaka problem med enzymets aktivitet och livstid, och dess effektivitet; problem medtillgängligheten till CO_2 på grund av begränsningar i diffusion och/eller masstransport, och då processen behöver laddas om med enzym. Vidare så förlitar sig fortfarande dessa processer på regenerering av lösningsmedlet vid höga temperaturer. De energifördelar som ges av att använda lågenergilösningsmedel i kombination med enzym går därmed förlorade. Av dessa anledningar har vi föreslagit en alternativ process i vilken enzymet är fritt löst i och cirkulerar med lösningsmedlet genom processen, och där regenerering av lösningsmedlet sker vid sänkt tryck. Förutom att

vara mindre energikrävande skulle detta innebära att regenereringen av lösningsmedlet sker vid en temperatur som enzymet tål.

Den regenereringstemperatur som rimligen kan nås är dock fortfarande för hög för de flesta kända karboanhydraser, och särskilt för de mest aktiva och effektiva. I detta projekt har vi därför utnyttjat några tidigare gentekniskt modifierade varianter av humant karboanhydras II som har fördelen av att vara fysiskt stabiliserade som del av ett försök som ville klargöra hur dessa enzym beter sig i olika aminlösningsmedel vid olika förhållanden, men liknande de som kan förväntas i den föreslagna processen. Från dessa experiment kan det fastslås att enzymer påverkas i olika hög grad av lösningsmedelstyp, då dessa är allt ifrån relativt denaturerande (MDEA), till bara svagt denaturerande (TEA), till att vara till och med stabiliserande (K_2CO_3). Det vill säga att i en praktisk process skulle användandet av TEA och/eller K_2CO_3 vara den naturliga utgångspunkten. Det kunde också konstateras att vid de analyserade kombinationerna av olika enzym/lösningsmedel/förhållanden så är det på gränsen att processen skulle kunna fungera. Å andra sidan återstår det många möjligheter till förändringar, och parametrar som kan justeras för att faktiskt kunna nå en fungerande process.

Parallellt konstruerades ytterligare två varianter av enzymet för att hitta modifieringar som skulle kunna vara fördelaktiga att inkorporera i de redan existerande varianterna. Båda dessa nya varianter hade ökad temperaturstabilitet, varav den ena erhöll en ökad smälttemperatur, från 17,7 °C till 76,7 °C genom en enda införd disulfidbrygga, och som då den kombinerades med redan existerande variant nådde en smältpunkt på 84,1 °C. Också den mindre termiskt stabiliserade andra nya varianten uppvisade intressanta egenskaper som indikerar att den antingen denaturerar mycket långsamt, eller har förmåga att helt återfå struktur och aktivitet efter termisk denaturering. Det är alltså mycket sannolikt att ett än bättre enzym skulle gå att konstruera genom olika kombinationer av olika varianter. I ett separat projekt gjordes också försök för att starta enzymproduktion i större skala i vilket inga uppenbara hinder påträffades. Det hade därmed gått att producera enzym i tillräckliga mängder för att ladda och testa en process i laboratorieskala, ifall projektet inte hade avslutats av andra skäl i förtid.

Sammantaget så anser vi att resultaten visar på att den förslagna processen skulle haft en rimlig chans att fungera som en lovande startpunkt för utveckling av en enzym-baserad och energieffektiv uppgraderingsprocess, där de redan tillgängliga modifierade eller vidareutvecklade enzymerna hade använts.

Summary

The highest added value of biogas is in the form of upgraded biogas for vehicles or injection to gas grid, in which the biomethane has been separated from the carbon dioxide of biogas. However, established methods for upgrading rely on high pressures or high temperatures and are thus very energy intense. Therefore, the high costs for upgrading has a very large impact on the economics of biogas production. Furthermore, the scalability of established upgrading techniques are low and are most often not suitable for smaller biogas plants. Both these circumstances impede the expansion of biogas production.

The research and efforts in process development for carbon capture and sequestration (CCS) has been much more intense. CCS basically deals with the same problems as the ones in biogas upgrading, i.e. to catch the CO_2 from out of gas mixtures, but with even stronger incentives to perform in an energy efficient manner, since no added value besides the environmental benefit is expected from capturing CO_2. Thus, all energy expenditure in CCS is from a cost perspective parasitic energy. Therefore very much resources and efforts are directed to research for finding alternative solutions for energy efficient CCS with low parasitic energy demand.

Some of the most efficient methods for CCS employ the use of amines as solvents for CO_2 of which some are however "too reactive" and makes very strong bonds with CO_2 and therefore consequently demand very high temperatures and energy to continuously regenerate the amine solvent. Less reactive solvents are available but the lower reactivity, with the corresponding lower rates of reaction, signify that they are not feasible for CCS because absorption towers would need to be very large/high and flow rates would need to be low in order to utilize the full CO_2 capacity of these solvents. In order to circumvent this reaction rate deficiency of low energy solvents, the use of enzymes (carbonic anhydrases) to increase rates of reaction has been suggested. However, for an enzyme-based CO_2 capturing process to work, all parts including the solvent, the process and the enzyme need to be considered and adjusted to be within each other's limitations. We have therefore in this project studied the literature and patents regarding research in all these areas with the intention to design and build an energy efficient enzyme-based biogas upgrading process in laboratory scale.

With regards to the process, several alternatives are presented in literature. All of these employ enzymes immobilized either in the absorption phase, or in both the absorption and desorption phase. This need to immobilize the enzyme will likely cause problems with activity and/or lifetime of enzymes; efficiency of the enzymes; problems with enzyme access to CO_2 due to diffusion and/or mass transport limitations, and enzyme regeneration of the process. Furthermore, the suggested processes still rely on regeneration of the solvent by heat and thus the energy benefit of using low energy solvents in combination with enzymes is thereby lost. For these reasons, we suggest an alternative process in which the enzyme is free to flow throughout the process with the solvent and in which the regeneration of the solvent takes place at decreased pressure, which in addition to be less energy demanding, allows the solvent to regenerate at a temperature which is low enough for the enzyme to withstand.

The regeneration temperatures that can reasonably be reached by decreased pressure are however still too high for most known carbonic anhydrases, and in particular the most active and efficient ones. In this project we therefore made use of some earlier engineered variants of human carbonic anhydrase II, that have the benefit of increased physical stability, in order to clarify how these react to different solvents at different conditions, but close to those that could be expected in the proposed upgrading process. From these experiments it can be concluded that the enzymes are influenced, to different degrees, by different solvents. Ranging from the fairly denaturing (MDEA), to mildly denaturing (TEA), to actually being stabilizing (K_2CO_3). Thus, in a practical process, TEA and/or K_2CO_3 would have been the natural starting point. It can further be understood that at the analyzed combinations of enzyme/solvent/condition, there is reasonable probability that the proposed process would have been feasible. It can further be understood that there are many possibilities and parameters that could be adjusted to increase that probability into certainty.

In parallel, two other variants of the enzyme were constructed in order to find alterations that might prove beneficial if combined with the already available engineered variant. Both these new variants had increased thermal stability of which one had the melting temperature increased by 17.7 °C to 76.7 °C by a single introduced disulfide bridge, and when combined with the already available engineered enzyme a melting point of 84.1 °C was reached. Also the less thermally stabilized variant displayed interesting features as initial experiments indicate that it either denatured very slowly, or had the ability to fully refold after thermal inactivation. Thus, it is very possible that an even better enzyme would have been possible to construct by combining different variants. In a separate project, efforts to set-up a larger scale of enzyme production was conducted in which no major obstacles was encountered. Thus it would have been possible to produce enough enzyme to load and run a laboratory scale equipment.

To conclude, it is in our opinion shown that the results suggest that a process as proposed would have had a high probability to have worked well as a starting point for developing an energy efficient enzyme based process for biogas upgrading, using the engineered enzymes available or further developed.

List of content

1 Background

Biogas is a renewable energy source which refers to gases produced by the breakdown of biological/organic matter under anaerobic conditions. The gas is usually produced in biogas reactors using either mesophilic digestion at moderate temperatures (20 – 45 °C), or thermophilic digestion (normally 50 - 55 °C). Organic raw materials for biogas production include food waste, sewage sludge, manure, agricultural, slaughter house and municipal waste. The main component of biogas is (bio)methane (CH_4, ~ 60%), a combustible gas that can be used as an energy source, and carbon dioxide (CO_2, ~ 40%), as well as trace amounts of other compounds, the chief one often being hydrogen sulphide (H_2S). In Sweden and in many other parts of Europe the highest value of biomethane is as automotive fuel. Present techniques for biogas upgrading are however very energy intense and the upgrading is a significant part of the operational costs of a biogas plant. In fact, upgrading of biogas to vehicle fuel standard constitutes the single largest energy consumption of high grade energy and can amount to approx. 1/3 of the production cost at a biogas facility that produces biomethane. Present techniques have also a low scalability, meaning that only plants producing above a certain threshold (approx. 50 Nm^3/h) may reach economic feasibility with current techniques available to upgrade biogas to gaseous automotive fuel standard, i.e. on par with natural gas. Development of more energy efficient and scalable biogas upgrading processes could therefore have a very positive impact on the economics of large scale biogas production facilities as well as opening up for automotive fuel production at e.g. farm biogas plants.

1.1 CO_2 SEPARATION FOR BIOGAS UPGRADING

The approximately 60/40 % (CH_4/CO_2) mixture of gases produced by anaerobic digestion is not of high enough quality to be used as a vehicle fuel. The CO_2, as well as the other pollutants, need to be removed so that the final product biomethane, contain at least 97 % CH_4, with the rest being only CO_2.

Some of the more established methods for upgrading biogas to vehicle fuel standard are based on pressure, including:

1. Water pressure *absorption*, based on the fact that CO_2 dissolves more readily than CH_4 in water at high pressure.
2. Pressure swing *adsorption*, based on the fact that CO_2 and CH_4 adsorbs with different strength to zeolites or active carbon at high pressure.
3. Physical (non-reactive) solvent adsorption, where acidic gases (such as CO_2) is dissolved in organic solvents (such as methanol, polyethylene glycol, and dimethyl ether) at elevated pressure.

The above and physically similar methods for biogas upgrading are well established and all have their respective strengths and weaknesses in terms of capacity, degree of purification, methane slip etc. However, they are all hampered by their high energy consumption due to high pressure for adsorption/absorption, which is the main reason to look at alternative up-grading technologies. Hence, these methods will not be further discussed in this report but comprehensive information can be found elsewhere [1, 2].

1.2 CHEMICAL ABSORPTION IN AMINE SOLUTIONS

Instead of the alternatives 1 - 3, the extensive knowledge and experiences from gas sweetening of natural gas and Carbon Capture & Sequestration (CCS) with alkyl alkanolamines and ethanolamines has been studied more in-depth as the overall objective is the same as in biogas upgrading, i.e. the separation of CO_2 from a mixture of gases. There is also currently a consensus view that the concentration of CO_2 in the atmosphere is the major contributor to increasing global warming, which has also been concluded by the Intergovernmental Panel on Climate Change (IPCC) [3]. Thus, several chemical methods have been suggested and tested for CCS. Globally, there are however only a few commercial installations for CCS today due to the high parasitic energy consumption of current methods. Nevertheless, the fast development of CCS, as compared to biogas upgrading technologies has resulted in that there are today approx. 54 large scale CCS projects in different development stages or in operation. A number that could not have been reached unless the energy consumption issue of CCS had to some degree been resolved [4].

In many of these post-combustion CO_2 capture projects, that includes CCS based on amine absorption, CO_2 is removed by a chemical absorption process that involves exposing a flue gas stream to an aqueous amine solution. The amines used are chemicals that can be described as derivatives of ammonia (NH_3) in which one or more of the hydrogen atoms has been replaced by an alkyl or alcohol group. Amines are classified as primary, secondary, or tertiary depending on whether one, two, or three of the hydrogen atoms of ammonia have been replaced by organic functional groups. The ability of an alkanolamine solution to remove acidic gases is determined by the acid gas solubility, the rate of reaction and the mass transfer properties [5]. Major methods for CCS is therefore amine gas treatment in which the CO_2 is captured by either:

A: Primary or secondary amines

Primary and secondary amines (such as monoethanolamine (MEA), diethanolamine (DEA) or diglycolamine (DGA) which involves a *direct* reaction between CO_2 and the alkanoamine which results in the formation of covalent carbamate bonds [6]:

$$2Am + CO_2 \Leftrightarrow AmH^+ + AmCOO^-$$

This reaction is reversible and the CO_2 can be released by heating the solution with the carbonate salt in a separate stripping column. Herein lies however the biggest drawback of using primary or secondary amines since these amines have high reactivity with CO_2 and therefore also tend to have high heats of reaction (≈ 80 kJ·mol^{-1} CO_2) due to the formation of the carbamate complex[6]. Thus, the reverse reaction and the extent of heat required to break down this complex during the regeneration stage will be high since it depends mainly on the heat of reaction. In practice, regeneration of the amine solution will need heating by water steam. This is a more manageable problem at for example an incinerator plant (for CCS) where steam would be available. However, at a biogas plant, additional investments in steam generators would be needed. Thus, in terms of using primary or secondary amines for CO_2 capture in biogas upgrading the investment and energy costs will be as high, or higher, than for the other upgrading methods listed in section 1.1.

Furthermore, in the carbamate mechanism, two moles of amine are required for each mole of reacting CO_2, meaning that primary and secondary amines have a maximum loading of 0.5 mol/mol. In practice and in most applications the loading is in reality

however less or equal to approx. 0.2 mol/mol due to amine degradation and other effects.

B: Tertiary amines

An alternative method is to use tertiary amines such as methyl diethanolamine (MDEA) or triethanolamines (TEA) which drives the formation of hydrogen carbonate ions (HCO_3^-) via an unstable form (carbonic acid, H_2CO_3) at a pH higher than 10 according to the reactions below:

1. (fast): $CO_2\,(g) \Leftrightarrow CO_2\,(aq)$
2. (slow): $CO_2\,(aq) + H_2O \Leftrightarrow H_2CO_3\,(aq) \Leftrightarrow H^+\,(aq) + HCO_3^-\,(aq)$
3. (fast): $R_3N + H^+\,(aq) + HCO_3^-(aq) \Leftrightarrow R_3NH^+ + HCO_3^-\,(aq)$

(Where: R= alkyl or alcohol)

Thus, since tertiary alkanolamines lacks the N-H bond necessary for the formation of carbamate ion the reaction of tertiary alkanolamines does not form carbamate species. Hence, it is not able to react with carbon dioxide directly and only involves a proton transfer mechanism which encourages the (still slow) hydrolysis of carbon dioxide in aqueous solution, to form bicarbonate (HCO_3^-) and protonated amine. The mechanisms involving tertiary amines should therefore be considered as a "hydrolysis catalyzed reaction", in which the alkanolamine works as a base that drives the reaction by capturing the proton formed upon solvation of CO_2, thereby shifting the equilibrium to the right . Because of this mechanism tertiary alkanolamines have higher capacity and lower heat of reaction (≈ 60 kJ·mol CO_2^{-1}). Therefore this reaction requires a correspondingly lower energy for regeneration, as compared to primary or secondary amines, as CO_2 is absorbed in the solution primarily as a bicarbonate (HCO_3^-). Another benefit of the tertiary alcohols is that they react at equimolar concentrations with CO_2 and thereby has higher capacity than the more reactive primary and secondary amines [7].

However, due to the slow rate of hydration of CO_2 into H_2CO_3 in the step preceding step 3 above, the overall rate of reaction for tertiary amines is considerably slower than for primary and secondary alkanolamines, with second order rate constants that is three order of magnitudes larger for primary amines (MEA and DEA) than for tertiary amines (MDEA and TEA) [8]. Therefore, for tertiary amines, it is the rate of reaction rather than solubility and mass transfer properties that is limiting process development since long residence times would be necessary in the absorber/desorber units (or simply translatable to very large/high absorption columns). Although the pK_a of all amines of interest is close to 10 by which they all provide solutions with pH above 10 (table 1), for the purpose of increasing rate of reaction, which in turn will influence the mass transfer efficiency and capacity per unit time, combinations of various primary, secondary and tertiary amines, are continuously evaluated. One such well-studied combination is to activate MDEA with piperazine which is known to accelerate the effect and enhance solubility of CO_2 in tertiary amines [8, 9].

Table 1 pKa and pH values of some selected amines [10]

Amine	pK_a @ 25 °C	pH of 10 % aq. solution
MEA	9.45	12.0
DEA	8.88	11.5
MDEA	8.52	10.7
TEA	7.77	10.2

Since the purpose of the tertiary amine solution based process is simply to provide a base that captures the proton upon hydrolysis of CO_2, in order to drive the equilibrium towards the hydrated state, also other basic solutions can be considered. One such example is solutions of the moderately strong potassium carbonate (K_2CO_3, $pK_a = 10.25$) which forms alkaline solutions. The use of other bases than amines will however not change the rate of reaction as it is still step 2 above that is rate limiting.

From the above parameters, and many more such as cyclic capacities, reaction heat generation (exothermic), mass transfer and solubility of CO_2 in various solution, it stands clear that the choice of amine solution will be very influential of outcome, both in efficiency (maximum methane content in the resulting biomethane), capacity (how much CO_2 can be captured per unit volume), and process/regeneration energy costs. All these outcomes are interrelated, e.g. a higher capacity of the amine solvent will lead to lower regeneration costs as a lower volume of solution needs to be regenerated by heating, but *only* if the high capacity amine solution has a low heat of reaction. Or another example, the efficiency could likely be increased by increasing the flow rate through the system or increasing absorber height, but this will be penalized through a larger volume of solution that need to be regenerated per unit time.

Furthermore, it must be kept in mind that the demands of the biogas upgrading application is very different to CCS. In CCS the sole purpose is to catch "as much CO_2 as possible" to the lowest cost possible. If this cost limitation is at 80 % CO_2 capture, that might still be OK, whereas in the application of biogas upgrading a prerequisite is that almost a complete capture of CO_2 is achieved to reach the 97 % methane of automotive fuel, while still be cost effective. Therefore not all information regarding the use of amines for CCS is transferable to the biogas upgrading application. In addition, in an enzyme catalyzed reaction also the properties of the enzyme in relation to the solvents and the process has to be considered. These properties cover a large range of parameters such as the enzymes intrinsic properties in catalytic efficiency and ability to withstand various extrinsic conditions such as ionic strength, polarity, pH, temperatures, shear forces, surfaces etc.

Due to the energy cost constrictions (regeneration heat) and the constrictions set by the properties of the enzymes (e.g. pH of solution) it would appear that primary and secondary amines are of less interest as solutions for enzyme based biogas upgrading and focus in this project has therefore been directed to tertiary amines.

1.3 ENZYME ACCELERATED REACTIONS IN TERTIARY AMINE SOLUTIONS

An alternative to increase the rate of reaction and the capacity of tertiary amines is to use enzymes (carbonic anhydrases, CA) that accelerate the rate of reaction of solubilizing CO_2 to proton (H^+) and carbonate (HCO_3^-) according to:

$$CO_2\,(aq) + H_2O \xrightarrow{[Enz]} H^+ + HCO_3^-$$

By this, the step 2 above is no longer slow and rate limiting, and all rates of reaction would be fast also for tertiary amines [11]. Furthermore, by this mechanism the tertiary amine would no longer *drive* the reaction but simply acts as a proton acceptor (base), allowing the enzyme to work efficiently in the forward reaction against a low concentration of protons without product inhibition according to:

$$R_3N(aq) + CO_2(aq) + H_2O \xrightarrow{[Enz]} R_3N^+H(aq) + HCO_3^-(aq)$$

This desired effect of carbonic anhydrases (CA) to solubilize CO_2 has been known for long and various process solutions to incorporate carbonic anhydrases for carbon capture and sequestration has been suggested, patented and published by many actors [12 - 18]

An enzyme catalyzed process could thus, theoretically, speed up the CO_2 capture reaction rate in tertiary amines, leading to a higher efficiency and capacity use of tertiary amines and thereby reducing the energy consumption per captured mass unit of CO_2 considerably in comparison to uncatalyzed processes in primary/secondary amines, respectively. All suggested processes generally operate by bringing CA, either free in solution or immobilized, in contact with CO_2 dissolved in the solution. However, neither of these processes is of any value if the necessary CA enzyme catalyst is not stable enough to function at the operational conditions or have long enough life-time to be economically viable.

1.4 BASIC OUTLINES OF ENZYME BASED CO_2 CAPTURE PROCESSES

A simplified schematic figure of an amine gas treatment plant is shown in Figure 1. The biogas basically enters the system in the bottom of the absorption tower where it is introduced to counter current flowing aqueous amine solvent (usually 30 - 50 % MEA or MDEA in water). The CO_2 is captured by the amine, either covalently (MEA) or in an acid-base driven reaction (MDEA), while the CH_4 passes through the tower unaffected and exits as biomethane at the top of the tower. The CO_2-rich solvent then enters the desorption tower where the CO_2 is released by heating the solution to approx. 100 - 120 °C, depending on which amine is used. The CO_2 is released at the top of the desorption-tower while the solvent is cooled and recirculated to the adsorption tower. Thus, the repeated heating to high temperatures and subsequent cooling of the solvent is the reason why the process is so energy consuming.

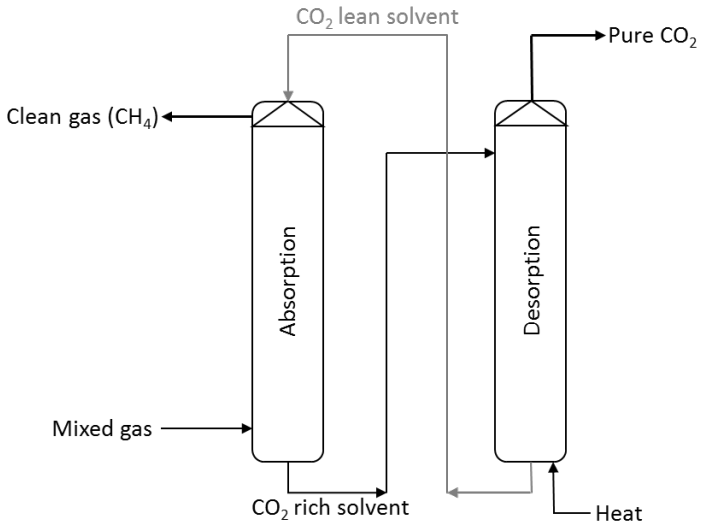


Figure 1 Simplified schematic of an amine gas treatment plant process.

If an enzyme were to be incorporated it could be done so in several ways:

1. In the absorption tower only, by which the enzyme must be retained either by filtering out and re-circulating the enzyme directly after the absorption column or by immobilizing the enzyme in the absorption column. For this the enzyme would need to be immobilized on particles/beads suitable for filtration or on the packing material of the tower. This presents several problems as an immobilized enzyme can lose activity and further lose or gain stability in the immobilization process. An additional problem is that if the enzyme is immobilized on the packing material the enzyme can become placed too far from the liquid/gas interface so that the CO_2 never reaches the active site of the enzyme within the residence time in the absorption tower. If this was the case the reaction would be rate limited by the mass transfer of CO_2 in the gas/liquid interface and in solution. Thus the effectiveness is very dependent on the viscosity of the liquid and wetting properties of the packing material to provide a very thin liquid surface. Furthermore, immobilization of the protein on the packing material could lead to a complicated process to replenish the enzyme with new enzyme once it is inactivated, which could on the other hand be simplified if the enzyme was immobilized on suitable particles.

   Furthermore, with the enzyme in the absorption tower only, the reverse reaction of the enzyme would not be utilized and the necessary high regeneration energy would remain. The two things that would be gained is that the reaction rate of absorption is increased so that a lower absorption tower could be used with remained efficiency and better use of the capacity of the tertiary amine (closer to saturation), leading to some higher energy efficiency as less volume of amine needs to be regenerated per unit captured CO_2.

2. In both the absorption and the desorption column. To retain the enzyme in the two separate columns would require the immobilization techniques discussed above with the associated pros and cons. However, in this setup the reverse catalyzed reaction of the enzyme could possibly be utilized, if the conditions are such that the

enzyme can effectively catalyze the reverse reaction. This will however require an extremely heat stable enzyme if it is to be retained in the desorption-column while the amine is to be regenerated by heat at above 100 °C. For this an enzyme with a thermal stability well beyond 100 °C would be needed, and there is to our knowledge yet no such enzyme available. Again, if the regeneration were to be performed at very high temperatures, the energetic gain would only come from that less volume need to be regenerated.

3. Free in the solution. In this case the enzyme would not need to be immobilized on surfaces/beads but flow free throughout and with the liquid. This would however put the enzyme under other stresses as it would come in contact with many different surfaces that could lead to surface induced denaturation of the enzyme. This would be especially significant at the, by necessity, very large gas/liquid interface in the absorption and desorption columns. The enzyme could also be exposed to high shear forces in pumps, valves, orifices etc. Furthermore, the enzyme would also be exposed to all conditions in different parts of the process such as the high/low temperatures, high/low CO_2 loading, high/low pH etc.

   Nevertheless, the benefits could also be manifold. Firstly, and although the enzyme would be exposed to all conditions, also the harsh conditions such as high temperature, they would be exposed to these for a limited time depending on flow rate and residence time in each part of the system. Therefore, if the residing time at e.g. high temperature could be limited enough, the life-time of the enzyme could be prolonged if the residing time at denaturing conditions is shorter than what it takes to inactivate the enzymes at that condition.

   Secondly, since the enzyme would not need to be immobilized, the amount of enzyme in the system would not be restricted to the amount that could be immobilized, due to area limitations, on e.g. the packing material. Thus, the efficiency and capacity could be regulated by the concentration of enzyme in the system. Thirdly, if the enzyme is allowed to circulate freely with the solution, rather than being immobilized, there would also be a more even distribution of enzymes in the liquid phase and thus a continuous access to enzymes close to the gas/liquid interface. Therefore the reaction rate could possibly not be rate limited by mass transport of CO_2 in the solution, but only by the diffusion rate of CO_2 over the gas/liquid interface.

From the above, and more parameters, it stands clear that regardless of what process is preferred or selected the demands on the enzyme will be very high. Not only should the enzyme possess a high enzymatic activity and efficiency to minimize enzyme load but also a high melting temperature, a high kinetic stability (basically to inactivate slowly at harsh conditions), withstand the tertiary amine solution, remain active and stable during immobilization, resist surface induced denaturation (especially at the gas/liquid interface) and more.

1.5 CARBONIC ANHYDRASES

The enzyme used to increase the efficiency of the carbon capture is carbonic anhydrases (CA, enzyme commission number 4.2.2.1). CA is a group of enzymes that catalyze the reversible reaction of carbon dioxide and water into bicarbonate and a proton, according to:

$$CO_2(g) + H_2O\ (l) \Leftrightarrow HCO_3^-(aq) + H^+(aq)$$

Carbonic anhydrases are widely distributed throughout nature and are categorized in five distinct classes, the α-, β-, γ-, δ-, and ξ-class [19]. The α-class CA can be found in vertebrates, bacteria, algae, and green plants whereas the β-class CAs are found in bacteria, algae, and chloroplasts. One of each type of δ- and ξ-class CA have been isolated from eukaryotic marine diatoms. The only γ-class CA (Cam) isolated so far has been isolated from the thermophilic Archaeon *Metanosarcina thermophile* [20]. However, since the five classes have evolved through convergent evolution they differ significantly from each other with regard to amino acid sequence, structure and activity.

The α-class CA belongs to a superfamily of homologous proteins *i.e.* their genes have evolved from a common ancestral gene. The α-CAs from vertebrates are among the most effective, with a turn over number (k_{cat}) of up to $1.4 \cdot 10^6$ s^{-1}, which is 10^7 times faster than the spontaneous reaction. Furthermore, the catalytic efficiency (k_{cat}/K_m) for *e.g.* human carbonic anhydrase II (HCA II) is $1.5 \cdot 10^8$ M^{-1} s^{-1}, which is close to a diffusion controlled reaction. Since one of the natural functions of CA in vertebrates is to facilitate the removal of CO_2 from blood it has been suggested that that CAs can be used as biological catalysts in bioreactors designed for capturing CO_2 from various gas streams.

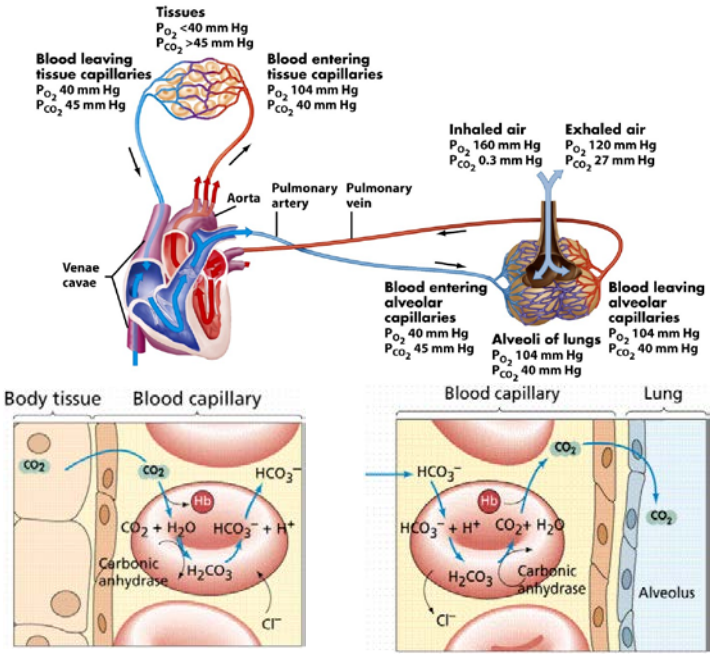


Figure 2 Biological function of HCA II and conditions at site of action.

Unfortunately, since there are no organisms living under the conditions similar to those in a CO_2-capturing bioreactor, nature has not provided us with a CA with the desired stability or efficiency. Mammalian, plant and eukaryotic CAs have through natural evolution been selected to be stable at physiological conditions of the respective organisms. Thus, α- and β-class CAs are generally only stable at approximately 37 °C or lower. Currently there are three heat-stable CA that has been isolated from nature.

A γ-CA (Mt-CamH) that has been isolated from *Methanosarcina thermophila*, an organism that has its optimal growth at 55 °C. Cam has a heat denaturation temperature midpoint (melting point, T_m) of about 70 °C, however, this enzyme has a catalytic turnover that is approximately 10-fold lower than that of HCAII (k_{cat} of approx. $1.2 \cdot 10^5$ s^{-1} as compared to $1.4 \cdot 10^6$ s^{-1}). Furthermore, the catalytic efficiency is approximately 20-fold lower ($7.5 \cdot 10^6$ M^{-1} s^{-1}) as compared to that of HCAII ($1.5 \cdot 10^8$ M^{-1} s^{-1}) [21]. Also important to understand is that Mt-CamH is a homotrimeric protein, *i.e.* an enzyme built up from three identical polypeptide chains, each containing 213 amino acids, and each monomer has to coordinate a Fe^{2+}-ion in order for the Mt-CamH to be in its most active form. This means that the protein must be produced anaerobically and be protected from air during purification and use.

The second reported thermostable CA is a β-class CA isolated from *Methanobacterium thermoautotrophicum* called Cab [22]. Cab is a tetrameric enzyme composed of four identical subunits each with a molecular weight of 21 kDa (173 amino acids) which is stable up to 75 °C, but has a k_{cat} that is approximately a 100-fold slower than that of HCA II ($1.7 \cdot 10^4$ s^{-1} compared to $1.4 \cdot 10^6$ s^{-1}) and a catalytic efficiency similar to that of Mt-CamH, $5.9 \cdot 10^6$ M^{-1} s^{-1}.

The third thermostable CA is a recently discovered α-class CA that was isolated from *Sulfurihydrogenibium yellowstonense YO3AOP1* (SspCA) [23]. SspCA have been show to retain activity up to 100 °C, and have a k_{cat} of $9.4 \cdot 10^5$, comparable to that of HCA II, but has a catalytic efficiency, $3.5 \cdot 10^7$ M^{-1} s^{-1}, that is roughly 10-fold lower that than that of HCAII. SspCA has been tested for carbon capture application reasons, where the enzyme were immobilized in polyurethane foam and its efficiency of retaining CO_2 in water were tested. The uncatalyzed reaction (column loaded with polyurethane foam) had an efficiency of 2.3% whereas addition of SspCA increased efficiency to 45%, in both cases the temperature was 25 °C [24]. Even though this experiment were neither performed in the same (or similar) solvent, nor at the same temperatures or pressures used in a conventional carbon capture unit, it could due to its high thermal stability prove promising for separation of CO_2 from flue gas in an industrial setting.

For the purpose of CO_2 capture also many other carbonic anhydrases have been identified or engineered with varying, and often only marginally, higher, thermostability than HCA II [25-29]. However, the conversion rate and efficiency is of great importance for the technical and economic feasibility of using CA in any CO_2-capturing process. Thus, if it would be possible to use HCA II, a bioreactor would require, roughly, 10 times less enzyme due to its high catalytic efficiency than a corresponding reactor using SspCA, the most promising of the thermostable CAs discussed above.

1.6 ENZYME STABILITY AND STABILIZATION

Enzymes are macromolecular protein biomolecules that are able to function as highly effective, high-performing biological catalysts and are fundamental for all known living

organisms. They are substances that accelerate the chemical reactions of life without themselves being consumed in the reaction. Isolated enzymes are important in many industrial processes for treating biological substrates. Thus, enzymes for industrial and environmental applications have a large and increasing economic and ecological value. One bottleneck in the application of enzymes in industrial processes is that in order to be active, enzymes must keep a highly ordered and folded structure. However, the highly ordered structure of proteins (including enzymes) is only maintained if the proteins are stable at the prevailing conditions *i.e.* pH, ionic strength, temperature *etc*., within certain limits that are specific for each type of protein. In terms of natural selection of proteins during evolution, this notion stresses the fact that a protein molecule only makes structural sense when it exists under conditions similar to those for which it was selected, in its so called native state. Protein stability can fundamentally be divided in chemical stability and physical stability.

Chemical stability relates to changes in activity of the enzyme in response to various chemical alterations, such as deamination of asparagine to aspartate and oxidation of methionine. Changes in activity can be due to changes of the amino acids involved in the enzymatic reaction or due to chemical modifications induces changes in or loss of structure leading to loss of enzymatic activity. Physical stability relates to the intrinsic ability of the protein to find and maintain its structure (and hence activity). Physical stability can be measured in several ways, e.g. as thermodynamic stability, the thermal stability and the kinetic stability which are all a function of the sum of interactions within the protein and between the protein and its surroundings.

Therefore, in the quest to design more stable proteins, it is important to understand the difference and benefits, as well as the underlying mechanisms, of each type of stability to be able to attain proteins with the desired increased stability. Thermodynamic stability is a measure of the difference in free energy (ΔG) between the unfolded (U) states and the folded state (F) in which the enzyme is active. Thermodynamic stability can be determined at the equilibrium conditions if the protein is free to unfold and re-fold. This two-state model can be written as:

$$F \rightleftarrows U$$

Thus, in this case the stability is simply the difference in free energy between the U and the F states ($\Delta G = G_{Unfolded} - G_{Folded}$) and the stability is defined as ΔG_{FU}, where

$$\Delta G_{FU} = -RT\ln K$$

K represents the equilibrium constant between the unfolded and the folded state (K=[U]/[F]) and, therefore, the more thermodynamically stable the protein is, the larger the difference in free energy (ΔG) is. This can also be graphically represented by plotting the difference in free energy between the unfolded and native state (Fig. 3)

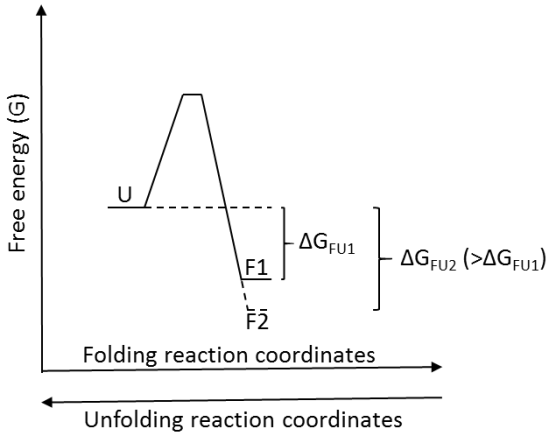


Figure 3 Illustration of the definition of difference in free energy (ΔGFU1) between the unfolded state (U) and the folded state (F) of a protein. Further, the graph illustrates how the thermodynamic stability can be increased by stabilizing the folded state ((ΔG$_{FU2}$).

Thus, simplified, the thermodynamic stability can be increased by either destabilizing the unfolded state (higher free energy of U) or by stabilizing the native state (lower energy of F) so as to maximize the difference in free energy (ΔG_{FU}) between the two states. The change in free energy needs to be lower than zero ($\Delta G < 0$) for the folding reaction to be efficient, that is, favoring the native state of the protein. Since the difference in free energy is determined by its enthalpy (ΔH, interactions) and the entropy (ΔS, disorder) according to $\Delta G = \Delta H - T\Delta S$, a favorable ΔG can be accomplished by strengthening the interactions of the folded state, leading to lowered enthalpy (*e.g.* more/stronger hydrogen bonds, ion bonds, better packing of the protein interior *etc.*). A larger difference in free energy between the unfolded and the folded state can also be accomplished by destabilizing the unfolded state. Furthermore, for the unfolded state, which can be assumed to be a random coil, the same can be accomplished by restraining the freedom of motion leading to lowered entropy and therefore a higher level of free energy of the unfolded state.

The melting point (T_m) of a protein, *i.e.* the midpoint temperature of unfolding, is a measure of a proteins thermal stability. In industrial processes it is often desirable to use enzymes with a high T_m since it is in many cases beneficial if the reaction can take place at an elevated temperature (higher rates of reaction, lower viscosity, less microbial growth, less fouling *etc.*). For this reason, what is often focused on for proteins that have a potential use in industrial processes is that the protein has a high thermal stability (*i.e.* high T_m). It is, however, important to recognize that at standard temperature (25 °C) the ΔG_{FU} values for a thermolabile protein are not necessarily lower than for a thermostable protein, *i.e.* a high thermal stability is not the same as a high thermodynamic stability at all temperatures[30]. Thus, it is not possible to deduce the melting point of a protein simply by determining its thermodynamic stability at ambient temperature or vice versa. The T_m is the temperature at which U and F are at equilibrium and are equally populated and is determined by the $\Delta G_{FU}(T)$ function, and will occur when the temperature is so high that $\Delta G_{FU} = 0$. When ΔG_{FU} is plotted as a function of temperature, the $\Delta G_{FU}(T)$ function is displays a skewed parabola that intersects the x-axis twice (i.e. both heat and cold denaturation can occur, Fig. 4).

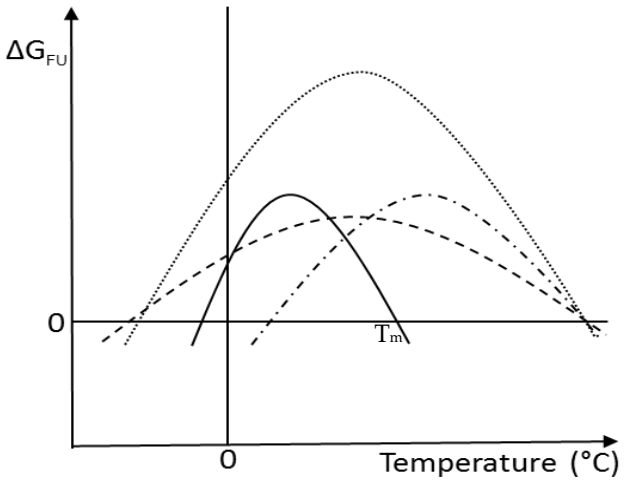


Figure 4 The relationship between thermodynamic and thermal stability, emphasizing that the knowledge of thermodynamic stability at a single temperature does not give any information about the melting temperature (T_m) of a protein. The $\Delta G_{FU}(T)$ function of a hypothetical thermolabile protein (—) with its melting temperature (T_m) and the possible increase in T_m by up shifting (……), right shifting (– • –), and flattening (- - -) of the function $\Delta G_{FU}(T)$.

Thus, thermal stability is related, but nor equivalent, to thermodynamic stability. That is, at ambient temperatures a protein can have a relatively low thermodynamic stability and still prove to have a relatively high T_m.

Kinetic stability is a measure of at what rate a protein unfolds (k_U). This is especially important for proteins that denature irreversibly, or when unfolding takes place at conditions that promotes irreversible denaturation. A protein can denature irreversibly if the protein in the unfolded state rapidly undergoes some permanent change such as proteolytic degradation or aggregation (which often is the case with thermally denatured proteins, e.g. the egg white of a boiled egg).

$$F \xrightarrow{k_U} U \longrightarrow \text{Irreversibly inactivated}$$

In these cases it is not the difference in free energy between the folded and unfolded state that is important. That will only affect the equilibrium and this is not a true equilibrium process. Instead, for kinetic stability, the important thing is the difference in free energy between the folded state (F) and the transition state ($ts^{\#}$) on the unfolding pathway which determines the activation energy for unfolding ($E_{A,\,unfolding}$). Hence, $E_{A,\,unfolding}$ determines the rate constant of unfolding (k_U) and thereby at what rate an irreversible inactivation of the unfolded state can take place (Fig. 5).

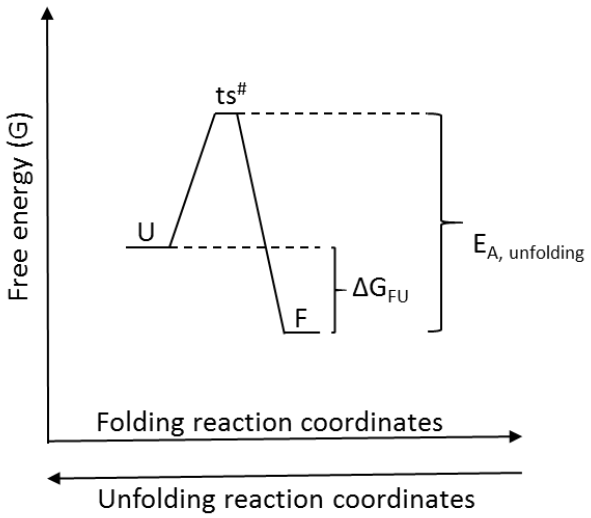


Figure 5 Illustration of the definition of activation energy of unfolding ($E_{A,\ Unfolding}$) of a protein determined by the difference in free energy between the folded state (F) and the transition state (ts#) on the unfolding pathway.

Thus, this is in no way related to the thermodynamic stability (ΔG_{FU}) or the thermal stability (T_m) and other means are necessary to increase the kinetic stability as compared to ΔG_{FU} and T_m. In order to change the free energy of the transition state the folding/unfolding mechanism of the protein needs to be affected. Simplified, when an ensemble of proteins fold they will mainly follow the fastest route that produces folding intermediates and transition states of lowest possible energy levels. However, if this route is no longer accessible, they will be forced to fold via a higher level of free energy than previously.

In this case, since the folded state has the same energy level as before (still needs to be in its highly ordered native fold to be active) the height of $E_{A,\ unfolding}$ will have increased and thus provide a barrier to unfolding leading to a slower unfolding rate constant (k_U) (and possibly also slower folding).

Therefore, for a protein to be valuable for any application it needs to have a large negative ΔG_{FU} at the temperature of operation so that the protein operates well below its T_m. Equally important is that it needs a high kinetic stability so that the protein is maintained in the natively folded state and the protein never end up in the unfolded state, which will often render it irreversibly inactive. Hence, a high kinetic stability will lead to slow unfolding and long lifetime of the protein. This is true for all conditions and will for example increase shelf life of the protein at ambient temperatures, but the activation energy for unfolding ($E_{A,\ unfolding}$) will also provide a barrier for unfolding also if the protein operates close to or even above its unfolding point (thermal or other) and thus keeping the unfolding rate constant (k_U) low and the lifetime high also at conditions that induce unfolding.

There are numerous ways of stabilizing proteins [31], either by stabilizing the folded state or by destabilizing the unfolded state by different means. However, most methods to stabilize the folded state rely on strengthening local interactions that are only formed once the protein is folded and few will substantially affect the folding route and hence the kinetic stability. One attractive way to stabilize a specific protein by knowledge-based engineering is to graft structural motifs that is known to be stabilizing from one

protein homolog to the protein homolog that is to be stabilized, of which there are numerous examples in the literature [32]. Two proteins are considered to be homologous if they have identical amino acid residues in a significant number of sequential positions along the polypeptide chain. However, the three-dimensional structure is much more conserved than the sequence and is it often found that proteins with very low sequence identity still have similar function and similar three-dimensional structures [33]. Thus, members of such families are also considered to be homologous even though polypeptide sequence identities are not statistically significant, only structurally or functionally significant. Furthermore, homologous proteins always contain a core region (structurally conserved regions) where general folds of peptide chains are very similar. That is, the scaffold of even distantly related homologous proteins with low sequence identity have similar structure. It is these relationships that make it possible to transfer stabilizing amino acid combinations or motifs between structurally homologous proteins if there is three-dimensional structural data available.

One way of reducing the freedom (*i.e.* entropy) of the unfolded state and thus place the unfolded state on a higher energy level is to introduce covalent links between parts of the protein. This can be done by changing the original amino acids to cysteines which are able to form covalent disulfide bridges (S-S) if the thiol groups of the two amino acid side-chains are correctly placed in space. To design such bridges is however not trivial since the geometry of an unstrained -CH_2-S-S-CH_2- bridge in proteins is limited to rather narrow conformational constraints, and deviations from the geometrical constraints will introduce strains into the folded structure. However, because of the geometrical constraints, identification of disulfide bridges are particularly amenable for homology modeling to identify amino acid positions to alter to cysteines in order to introduce disulfide bridges in homologous proteins, of which there are numerous examples of in the literature [34]. Although this method has a limited rate of success since the replacement of the wild-type amino acid and the introduction of a disulfide bridge will often lead to loss of other favorable interactions or strain in the folded state, it will lead to a larger thermodynamic stability (ΔG_{FU}) if the folded state is unaffected (Fig. 6).

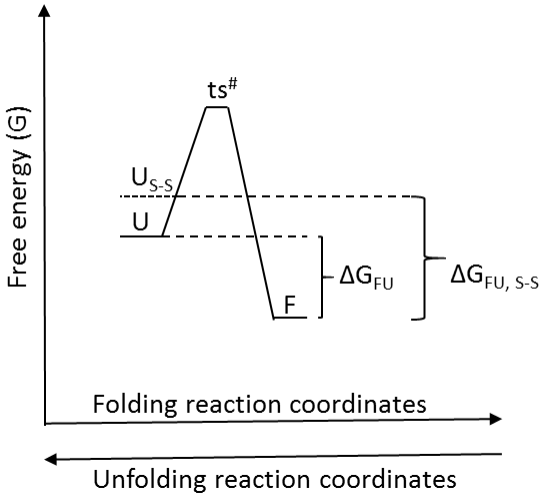


Figure 6 Illustration of how the thermodynamic stability (ΔG_{FU}) for a protein is increased by restricting the freedom of the unfolded stat by incorporation of a disulfide bridge ($U_{S\text{-}S}$), thus placing the unfolded state on a higher energy level.

Further, if the introduced disulfide bridge brings together parts of the protein that normally are in close contact during early stages of the folding event, it will not normally affect the folding pathway and will thus only increase the thermodynamic stability and possibly the rate of folding (under the prerequisite that the energy level of the folded state is unaffected). If however the introduced disulfide bridge bring parts of the protein together, that during normal folding does not interact early in the folding event, this will lead to that the protein likely needs to fold via an alternative route that has a transition state of higher free energy. Under the prerequisite that the energy level of the folded state is unaffected, this will lead to that the activation energy for unfolding ($E_{A,\ unfolding}$) will become higher and thus the unfolding rate will be slower and the lifetime of the protein will be increased. If this can be accomplished, an ideal protein, with both a high thermodynamic stability (and possibly increased melting temperature) and a high kinetic stability is constructed (Figure 7).

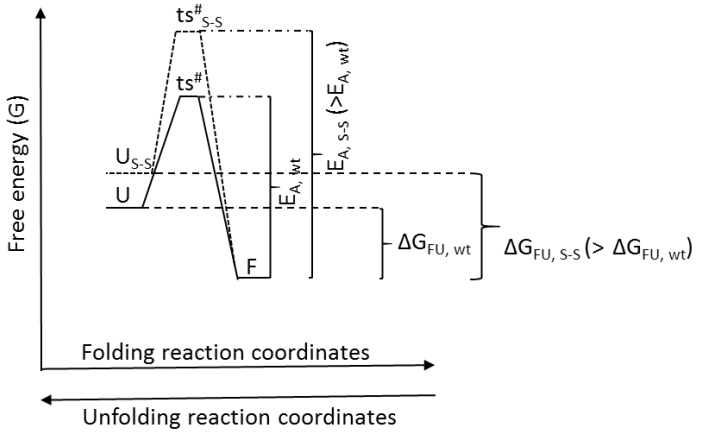


Figure 7 Illustration of the resulting increase in both thermodynamic stability ($\Delta G_{FU,\ S\text{-}S}$) and the activation energy ($E_{A,\ S\text{-}S}$), for a protein with a disulfide bridge inserted at a position that affects both the freedom of the unfolded state as well as the folding pathway and thereby the transition state (dotted line)). Comparison is made with an unmodified reference wild-type (wt) protein (solid line).

Besides being potentially able to increase both the thermodynamic and the kinetic stability of proteins, the stabilization is of entropic origin by restricting the freedom of the unfolded state by incorporation of a covalent bond (disulfide bridge). Thus, enthalpic stabilizing interactions by introducing disulfide bridges will not display a strong temperature dependence, which can otherwise weaken or strengthen *e.g.* hydrogen bonds, salt bridges, ionic bonds or hydrophobic effects. In addition, this also means that the stabilizing media, such as polarity and ionic strength *etc.*, and the relative increase in stability will be maintained also in media other than buffered aqueous solutions.

1.7 ENGINEERED STABILIZED HUMAN CARBONIC ANHYDRASE II

Since there are no naturally occurring carbonic anhydrases meeting the requirements that need to be met to be used in an enzyme based bioreactor to capture CO_2, there exists a need in the art of protein engineering to develop a CA that meets the expected requirements. That is, that are simple and economical to produce, have a high catalytic activity, have a high physical stability, and a long lifetime under various conditions. As noted, several such carbonic anhydrases has been discovered and developed [25-29]. However, all have had their limitations in general properties or for the process solution

they have been suggested. Therefore, and based on theory above, a modified human carbonic anhydrase II was earlier constructed, characterized and patented [35]. The enzyme is based on two introduced disulfide bridges of which one gives a kinetic stability [35] and the other gives the enzyme a high thermodynamic stability [36] and was later also found to provide the enzyme with increased pH stability [37]. By combining the two disulfide bridges the enzyme was shown to have both an increased thermodynamic and kinetic stability, as well as an increased thermal stability (table 2), while retain the high activity of human carbonic anhydrase to approx. 80 %.

Although this is the human carbonic anhydrase II with the highest thermal stability known, the enzyme does still not have a high enough thermal stability to withstand amine regeneration at above 100 °C, thus excluding its possible use in amine based upgrading processes as described in section 1.1. Furthermore, since the melting of a protein is not digital but takes place over a span of approx. 10 °C the regeneration temperature would need to be brought down to about 70 °C or lower even for the stabilized variant.

Table 2 Stability parameters of natural and engineered human carbonic anhydrase II.

Stability parameter	HCA II	Engineered HCA II
Thermodynamic stability, aqueous solution @ 23 °C (kJ/mol)	30.5	54
Melting point in aqueous solution (°C)	59	78
Activation energy for unfolding @ 23 °C (kJ/mol)	96	121
Unfolding rate in aqueous solution @ 23 °C (min^{-1})	$9.1 \cdot 10^{-5}$	$4.2 \cdot 10^{-9}$
Times slower unfolding		22 000

1.8 PROPOSED PROCESS

Based on all the above regarding the mechanism and regeneration energy of amines, and the basic process and limitations of available enzymes, an alternative process was proposed, based on desorption and regeneration of a solution of tertiary amines at lowered pressure, rather than at very high temperatures, and with the enzyme free in solution. Such a process would have many benefits.

1. The regeneration heat of tertiary amines are considerably lower than for primary amines. Whereas a process based on primary amines basically does not need to be catalyzed, the reaction rate of tertiary amines is low and in need of a catalyst to be as efficient. However, if available, such a catalyst could significantly reduce regeneration energy costs.

2. The amount of energy to lower the pressure over the desorption column is less than continuously heating and cooling the flowing volume of amine solution between approx. 30 °C (in absorption column) to above 100 °C in the desorption column.

3. This is also a technically feasible process as has been shown by Purac Puregas AB in their CApure process. In the CApure process the MDEA is brought to boiling

and regeneration at approx. 80 °C by lowering the pressure to approx. 500 mbar. This is made possible since the boiling point of binary solutions of MDEA/water is very similar to that of pure water up to approx. 50 % MDEA [38]. At mixtures of above 60 % MDEA, the boiling point of MDEA/water solutions then rises steeply.

However, if it were possible to lower the pressure even more than what is done in the CApure process it would be possible to reach a lower boiling point which the modified enzyme would withstand. At e.g. a pressure of 0.25 bar the boiling point would be approx. 65 °C, i.e. well below the melting point of the modified HCA II.

4. By bringing the solution to boiling the mass transfer of CO_2 is considerably enhanced simply because the much larger surface that is the result of the boiling liquid.

   A forced ventilation of the desorption column would also allow the enzyme to function more in line with its biological function, in which the direction of the reaction is dependent on the partial pressure of CO_2 (see fig. 2), since at desorption and for the enzyme to catalyze the reverse reaction (i.e. to the left),

   Diffusion [Enz]

   $$CO_2\,(g) \Leftrightarrow R_3N(aq) + CO_2\,(aq) + H_2O \Leftrightarrow R_3N^+H\,(aq) + HCO_3^-\,(aq)$$

   the CO_2 must continuously be withdrawn from out of the liquid phase. That has nothing to do with the catalytic reaction itself, but only the mass transport and diffusion rate of CO_2 out of the solution which is proportional to, surface area · concentration gradient / distance.

   Thus with a small surface area and a large distance to the gas/liquid interface the diffusion will be slow. In the lungs these problems are counteracted by the pulmonary alveoli, which is the gas exchange surface of the lungs with a surface area of approx. 70 m^2 (about half a tennis court), and a very thin liquid film covering these. Thus, the surface area and wetting of packing material and distance between enzymes and the gas/liquid interface is equally important at desorption as it is in absorption.

5. By keeping the enzyme free in solution there will always be enzyme present close to the gas/liquid interface, thus facilitating both absorption and desorption of CO_2 in the process.

   This necessitates that the enzyme has a high enough thermal stability to withstand the temperatures in the desorption column (i.e. approx. 65 °C in the proposed process), which is fulfilled by the engineered enzyme. However, it further necessitates that the enzyme withstands interactions with various surfaces and interfaces throughout the process. This has earlier been shown to be the case for the enzyme with one of the constituting disulfide variants of the enzyme, which confer high thermodynamic stability to the enzyme [39]. For this stability at surfaces, and also stability against e.g. shear forces, it is likely that also the very high kinetic stability of the enzyme will play a part to increase the lifetime of the enzyme, as there are indications that kinetic stability is just as important for this property [40].

6. If such a catalyzed process were to be realized it would also increase the efficiency and capacity, as compared to uncatalyzed processes. This is so because if rate of reactions were increased, lower absorption and desorption columns would likely be needed to capture and release the same amount of CO_2. Furthermore, the CO_2 capturing capacity of tertiary amines would likely be better utilized. From practical experiences it has been found that only about 20 % of the capacity of MDEA is exploited in uncatalyzed processes. With an enzyme catalyzed process this number would likely increase significantly because it is no longer the tertiary amine driving the (slow) reaction, but it only acts a proton acceptor for the (fast) reaction of the enzyme. Therefore less volume of amine solution would be needed (in theory 1/5 at 100 % saturation of MDEA) leading to a further drop in energy costs for heating/cooling.

It is obvious that "all parts affect all parts" and the possibilities of the above proposed process e.g.:

- Amines (capacity, heat of reaction, concentration, influence on protein stability, viscosity etc.)
- Process (temperatures, flow rates/residing times, pressures, packing material, wetting/liquid films etc.)
- Enzyme (activity and stability in amines, parts of process, concentrations etc.)

All these parameters are further intertwined and a change in e.g. amine concentration will influence how the process needs to be operated (e.g. flow rates), but will also influence the stability of the protein. This is made up of far too many unknowns in order to theoretically find optimal process parameters, so the only feasible way is to construct a laboratory process in which all parameters could be regulated, monitored and tuned.

Such a laboratory equipment was designed in collaboration between Purac Puregas AB and InZymes Biotech AB with the support of the equipment provider SAXE Nordic and their supplier Normag Labor- und Prozesstechnik GmbH (see section 3.4). This was however not realized because with the necessary specifications the cost would have been much higher than initially indicated and exceeded budget by 25 %. This was the main reason for the premature ending of the project.

2 Experimental

The experimental parts were conducted in parallel with literature studies and process design.

- At Linköping University studies were made to (*i*) analyze the engineered variant of HCA II activity and stability in amine solutions at different conditions, close to these expected in a process. Attempts were also conducted to (*ii*) increase the physical stability even further by structural analysis and site-directed mutagenesis for introduction of additional disulfide bridges to the already stabilized variant of the enzyme.
- At Medicago AB in Uppsala methods to produce larger quantities, enough for loading and evaluating a laboratory scale process, of modified HCA II were developed and evaluated. This work was conducted mainly in Q1 and Q2 of 2014 and thereafter put on hold when it was clear that a laboratory process would be delayed.

2.1 ENZYME ENGINEERING, PRODUCTION & ANALYSIS

2.1.1 Design of new disulfide bridge HCA II variants

To identify possible locations to introduce alternative disulfide bridges the three-dimensional structure was analyzed in the program SSBOND using X-ray structural data from the research collaboratory for structural bioinformatics (RCSB, protein data bank id. 2cba) by which 59 possible disulfide bridge locations were identified. The majority of these would produce disulfide bridges with very few amino acids between the cysteines and hence a low entropy decrease of the unfolded state and only marginally thermodynamic stabilization of the enzyme, provided that the free energy of the folded enzyme was unaffected. Therefore only those alterations with > 50 amino acids between the cysteins of a disulfide bridge were further considered.

To find if the positions suggested for cysteines in HCA II had in any case a corresponding homologous enzyme with a cysteine in the suggested position, the amino acid sequence of HCA II was then compared against other naturally occurring α-carbonic anhydrases, including sequences from uncharacterized organisms (metagenomes and transcriptomes) using BLAST. Several such positions were identified. In order to maximize the possibility of also reaching a high kinetic stability, the positions for introducing cysteines were then narrowed down to those that were judged to bind together parts of the structure that are formed late in the folding, which is normally outside of the hydrophobic core of the enzyme. Finally, the positions in which the introduced cysteine replaces an amino acid of similar properties (size, polarity/charge etc.) were identified in order to prevent strain in the folded state.

Several candidates were identified and two were constructed, produced and analyzed (L60C/S173C and D41C/L257C)

2.1.2 Plasmid preparation and site-directed mutagenesis

The plasmid pACA carrying the gene for HCAII pseudo wild-type with the modification C206S, i.e. with a cysteine replaced by a serine in position 206 (from here on designated $HCAII_{pwt}$) and the genetically engineered and physically stabilized

HCAII pwt A23C/L203C/S99C/V242C (from here on designated $HCAII_{Eng3}$) was isolated from a stock of *Escherichia coli* (*E. coli*) using a Qiaprep Spin Kit (Qiagen).

For additional modifications, the purified plasmid was then used for site-directed mutagenesis (QuikChange II XL Kit, Agilent Technologies) to introduce cysteines in position L60 and S173, and D41 and K257 respectively in $HCAII_{pwt}$, and in $HCAII_{Eng3}$ to produce new variants. Successful mutagenesis was verified by DNA sequencing performed by GATC GmbH.

2.1.3 Enzyme production and purification

Cultures of *E. coli* carrying the vector pACA encoding the genes for

- $HCAII_{pwt}$ or:
- Single disulfide bridge variants: A23C/L203C (Eng_1), S99C/V242C (Eng_2)
- L60C/S173C (Eng_4), D41C/K257C (Eng_5).
- Double disulfide bridge variants: A23C/L203C/S99C/V242C (i.e. Eng_3).
- Triple disulfide bridge variant: A23C/L203C/S99C/V242C/L60C/S173C (Eng_6).

were grown at 37 °C and protein expression were initiated by addition of IPTG (Isopropyl β-D-1-thiogalactopyranoside) and $ZnSO_4$ to a final concentration of 0.5 mM of each. The cells were harvested after three hours of induction by centrifugation and the pellet reconstituted in 0.1 M TRIS-H_2SO_4, 0.2 M K_2SO_4, pH 9.0 and thereafter lyzed by ultra-sonication. The cell lysate were then separated from cellular debris by centrifugation 11 000 × g.CO_2 activity, a clear indication of successful HCAII expression, of the lysate were then measured by mixing 30 µl lysate + 3 ml Veronal buffer (25 mM Veronal-H_2SO_4 and 50 mg/ml of the pH indicator bromothymol blue, pH 8.2) with CO_2 saturated water on ice and measuring the time it takes for the solution to reach pH 6.5, as indicated by the color change of the pH indicator from blue to light green. The HCA II variants in the respective lysate were then purified by affinity chromatography against a benzensulfonamide ligand coupled to an agarose gel and eluted against 0.5 M Sodium azide. The eluted protein were dialyzed against several volumes of 20 mM sodium phosphate buffer, pH 7.5. The final protein solution was thereafter concentrated in centrifugal filters (MWCO 10 kDA) to a final concentration of above 0.5 mg/ml.

To verify formed disulfide bridges, the protein were then analyzed for detection of free thiols, which, if present, indicates unsuccessfully formed disulfide bridges. This was done by mixing 17 µM protein in 0.1 M TRIS-H_2SO_4, 5 M GuHCl, pH 7.5 with 3.42 µl 50 mM 4-Chloro-7-nitrobenzofurazan (NBD-Cl) and measuring the change in absorbance at 420 nm. The variants that had remaining free cysteines were oxidized with 3 mM dithiothreitol and 1 mM *trans*-4,5,-Dihydroxy-1,2-dithiane for 96 h at room temp. or 48 h at 37 °C.

2.1.4 Near-UV circular dichroism (Near-UV CD)

Circular dichroism in the Near-UV wavelength spectrum basically reports on the purity and the overall folded state of the protein by presenting a unique "fingerprint" spectrum for different proteins. That is, a mixture of protein of badly purified protein will present a mixed spectrum and an unfolded protein will provide no spectrum. The state of the protein can thereby be monitored. In order to analyze the effect of different mixtures of MDEA, at different temp., Near-UV CD spectra were recorded on a Applied Photophysics Chirascan, by scanning the protein (24 µM) between 250 – 320

nm in 20 mM sodium phosphate, pH 7.5 as well as in mixtures of the buffer with MDEA (2, 5, 10, 25, and 50 %). The effect of increasing temperatures was measured by collecting a wavelength scan of the pure enzyme after 10 minutes of incubation at 40 °C. The temperature was then increased to 65, 70, or 75 °C and another scan was collected after 10 minutes of incubation at each target temperature. This was followed by cooling the sample to the starting temperature of 40 °C, incubating for 10 minutes, and collection of a final wavelength scan after the complete heating/cooling cycle.

2.1.5 Differential Scanning Calorimetry (DSC)

Protein melting temperature (T_m) was measured using DSC. Temperature scans were collected, starting at between 20 – 40 °C and ending between 85 – 100 °C depending on which mutant were investigated. Measurements were performed on 500 µg protein in 20 mM sodium phosphate, pH 7.2 as well as in mixtures of the buffer and MDEA or TEA (2, 5, 8, 10, 12, 14, 16, 18, 20, 25, or 50 %). The data were then analyzed with the program CpCalc.

2.2 ENZYME PRODUCTION (MEDICAGO AB)

2.2.1 Codon optimization and cell bank construction

DNA codon optimization and plasmid preparation for production of the HCA II variant HCA II_{Eng3} in *E.coli* was performed on contract by DNA 2.0, Inc. The plasmid were then transformed into *E. coli* and a small scale protein expression and purification was performed as earlier described before the activity of the protein was measured, as described above. The transformed cells were then used to create a cell bank.

2.2.2 Pilot scale protein expression

Cells from the cell bank were used to make a large scale culture optimization in a 13 l bioreactor, using Medicago's standard culture medium. This would, if successful yield at least 10 g of enzyme per reactor. The expressed protein was purified by affinity chromatography.

3 Results and discussion

3.1 CHARACTERIZATION OF VARIANTS OF HCAII

To make the results and discussion easier to follow the HCA II variants discussed have been assigned a short abbreviation as listed in table 1.

Table 3 Enzyme nomenclature for results and discussion

Enzyme variant	Abbreviation
HCAII pseudo wild-type	$HCAII_{pwt}$
HCAII A23C/L203C	$HCAII_{Eng1}$
HCAII S99C/V242C	$HCAII_{Eng2}$
HCAII S99C/V242C/ A23C/L203C	$HCAII_{Eng3}$
HCAII L60C/S173C	$HCAII_{Eng4}$
HCAII D41C/K257C	$HCAII_{Eng5}$
HCAII S99C/V242C/ A23C/L203C/L60C/S173C	$HCAII_{Eng6}$

Of these it is the double disulfide HCA II_{Eng3} that is the original modified enzyme that possibly has the properties suitable for a CO_2 capturing process, as described in table 2 and constructed by a combination of the single cysteine variants of HCA $II_{Eng1\ \&\ Eng2}$. That is, with a thermodynamic stability increased by 23.5 kJ/mol, an activation energy of unfolding increased by 25 kJ/mol and a thermal stability increased by 18.5 °C. HCA $II_{Eng\ 4\ and\ 5}$ are additional single disulfide variants created within this project, of which one (L60C/S173C) was incorporated into the original modified HCA II_{Eng3} to create the new triple disulfide variant of HCA II_{Eng6}.

3.1.1 Characterization of HCA II_{Eng3}

To further characterize HCA II_{Eng3}, and to get a starting point for analyzing the enzyme in conditions more resembling those that are encountered when used in an amine gas treatment plant, the enzyme were analyzed using DSC and the results were compared to those for $HCAII_{pwt}$, as well as for the two single disulfide variants of HCA II_{Eng1} and HCA II_{Eng2}. Thermal unfolding of the four HCAII variants were measured in a 20 mM sodium phosphate buffer, pH 7.5. The pseudo wild-type (HCA II_{pwt}) had its midpoint of thermal unfolding at 58.6 °C, as can be seen as the peak (solid grey line) in fig. 7. The introduction of disulfide between a cysteine in position 99 and 242 in HCA II_{Eng1} has earlier been shown by fluorescence measurements to increase the thermal stability of the enzyme, this was corroborated by the DSC measurement performed here, where the thermal unfolding occurred at a higher temperature (65.1 °C) than for HCA II_{pwt}. The second variant of the enzyme, containing a single disulfide bridge, HCA II_{Eng2}, have also been shown to have an increased thermal stability, above both HCA II_{pwt} as well as HCA II_{Eng1}. This is also verified in this study by DSC in which HCA II_{Eng2} has its unfolding transition midpoint at 70.6 °C (black dashed line in Fig. 7).

When the thermal stability of HCA II_{Eng3} was measured it is obvious that the stabilizing effect of the two single disulfides are cumulative since the double disulfide bridge variant HCA II_{Eng3} has its thermal unfolding midpoint at 77.6 °C (solid black, Fig. 8).

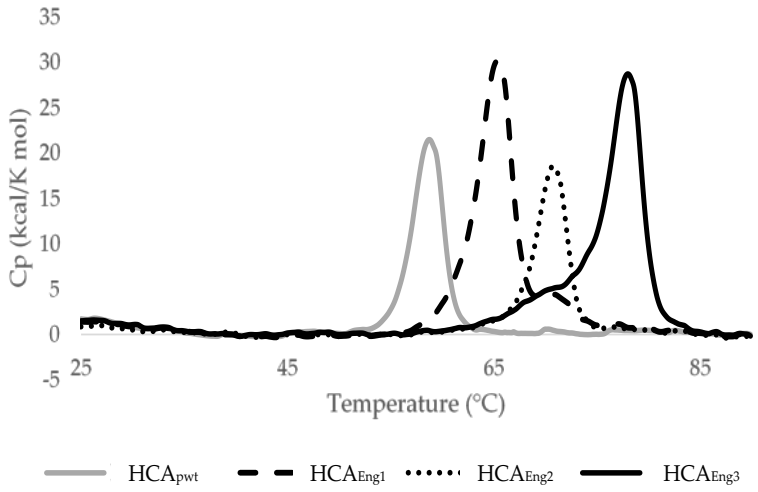


Figure 8 Thermograms showing the specific heat as a function of temperature of the four HCAII variants HCA II_{pwt} (solid grey line), HCA II_{Eng1} (dashed black line), HCA II_{Eng2} (dotted black line), and HCA II_{Eng3} (solid black line).

Unfortunately, the results point to that thermal unfolding of HCA II variants is irreversible (fig. 9). This thermogram shows the data for specific heat of a complete heating/cooling cycle, in which the cooling of the HCA II_{Eng3} solution is performed at the same rate as the temperature was increased when determining the thermal unfolding. This figure shows the actual data of the thermal unfolding (grey dotted line) and the thermal refolding (black solid line), showing no generation of heat from refolding. As an example of how the thermogram would have looked if the enzyme could refold completely upon cooling, there is for comparison a theoretical grey dashed line. This indicates that when the enzyme is used in any application the temperatures used cannot be allowed to exceed the T_m, since this would render the enzyme irreversibly inactive.

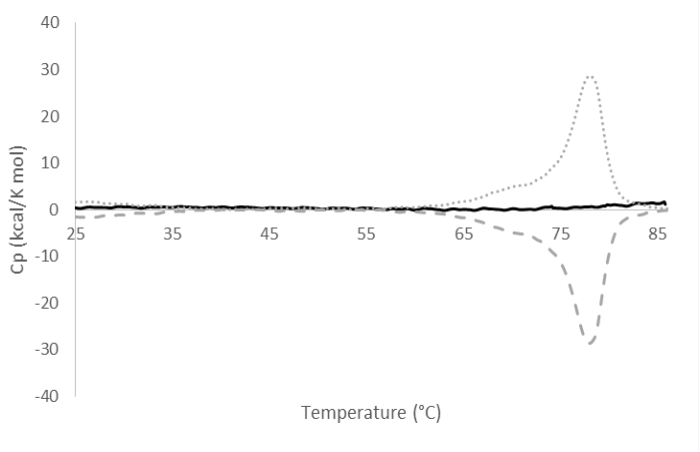


Figure 9 Thermogram of the irreversible thermal unfolding of HCA II_{Eng3}. The grey dotted line shows the behavior of HCA II_{Eng3} upon heating and the black solid line shows the behavior upon subsequent cooling. The dashed grey line shows an example of how the enzyme would behave if it were refolding with decreasing temperature.

Note, that for HCA II_{Eng3} (with a thermal unfolding midpoint of 77.6 °C) the melting curve is not an even parabola and there is some absorption of heat between 55 and 70 °C. This is most likely not a behavior of the HCA II_{Eng3} variant itself but an artefact due to small amounts of the enzyme variant not having formed both the disulfide bridges, which thus has a lower Tm than the majority of correctly formed HCA II_{Eng3}. In either case, the thermogram displays the normal behavior in that thermal unfolding is not "digital" but takes place over a rather broad temperature interval.

For an enzyme that unfolds irreversibly, that temperature interval cannot be entered at all because that would lead to that small amounts enzyme is continuously being inactivated until all enzymes are inactivated and irreversibly lost. Thus the upper working temperature of an enzyme is not the Tm, but approx. 5 – 15 °C below the Tm, depending on the width of the melting curve. For the HCA II variants that working temperature would appear to be somewhere between 12 – 15 °C below Tm, or 62.6 – 65.6 °C. That is, close to the boiling point at 0.25 bar as explained earlier.

3.1.2 Stability of HCA II_{Eng3} in the presence of MDEA

An amine gas treatment biogas upgrading plant of course utilizes amines, which are in fact organic solvents and in e.g. Purac puregas' CApure process, 50 % (v/v) MDEA is used to enhance CO_2 capturing. The natural environment for an enzyme is a buffered aqueous solution, and since it is well known that enzymes and proteins do not generally have a high stability in organic solvents, we investigated the thermal stability of HCA II_{Eng3} in the presence of MDEA. The thermal unfolding of HCAII_{Eng3} in 2, 5, 10, 25, and 50 % MDEA were investigated using DSC (Fig. 10). As shown above, HCA II_{Eng3} has a T_m of 77.6 °C in a buffered water based solution. However results show that by adding just 2 % MDEA the T_m decreases by 3.7 °C, to 73.9 °C.

Increasing the amount of MDEA further has an increasingly negative effect on the T_m, at 5 % MDEA T_m is 71.0 °C, at 10 % MDEA T_m is 66.3 °C, at 25% MDEA T_m is 62.1 °C, and at 50 % MDEA it has dropped down to 57,0 °C which is even 1.5 °C below the T_m of wild-type HCAII in plain buffered aqueous solution.

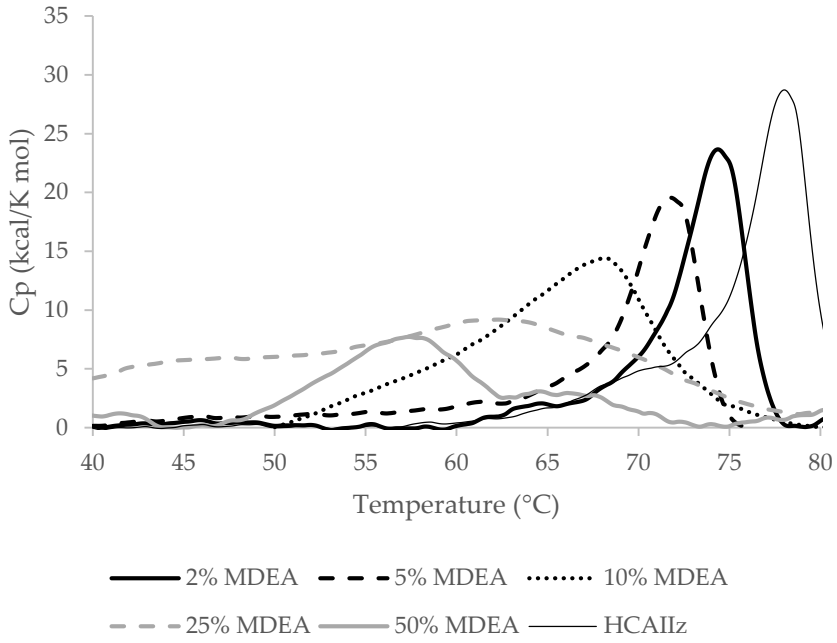
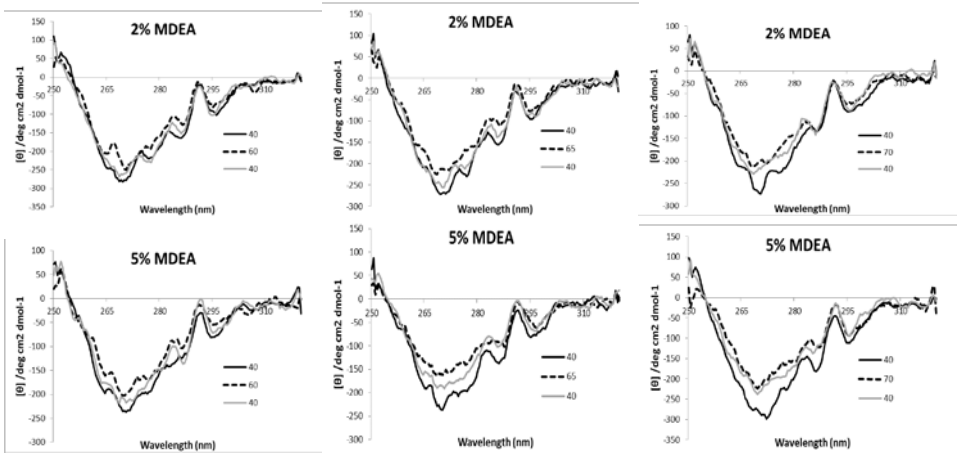


Figure 10 Thermograms showing the specific heat as a function of temperature of 500 µg HCA II_{Eng3} in buffer (thin black line), 2 % MDEA (solid black line), 5 % MDEA (dashed black line), 10 % MDEA (dotted black line), 25 % MDEA (dashed grey line) and 50% (solid grey line).

A similar thermal stability experiment were performed using CD-spectroscopy. In this experiment HCA II_{Eng3} was incubated with solutions containing MDEA (2, 5, 10, 25, and 50 %) for 10 minutes at 40 °C before a spectrum was recorded between 250 – 320 nm. The temperature was then increased to 60, 65, or 70 °C and incubated at that temperature for 10 minutes before another scan was performed. This was followed by a subsequent cooling, back to 40 °C, where the sample once again was incubated for 10 minutes prior to a final scan (Figure 11). This experiment was performed as to mimic the time the enzyme would be subjected to the different temperatures in the absorption unit (~ 40 °C) and the desorption unit.



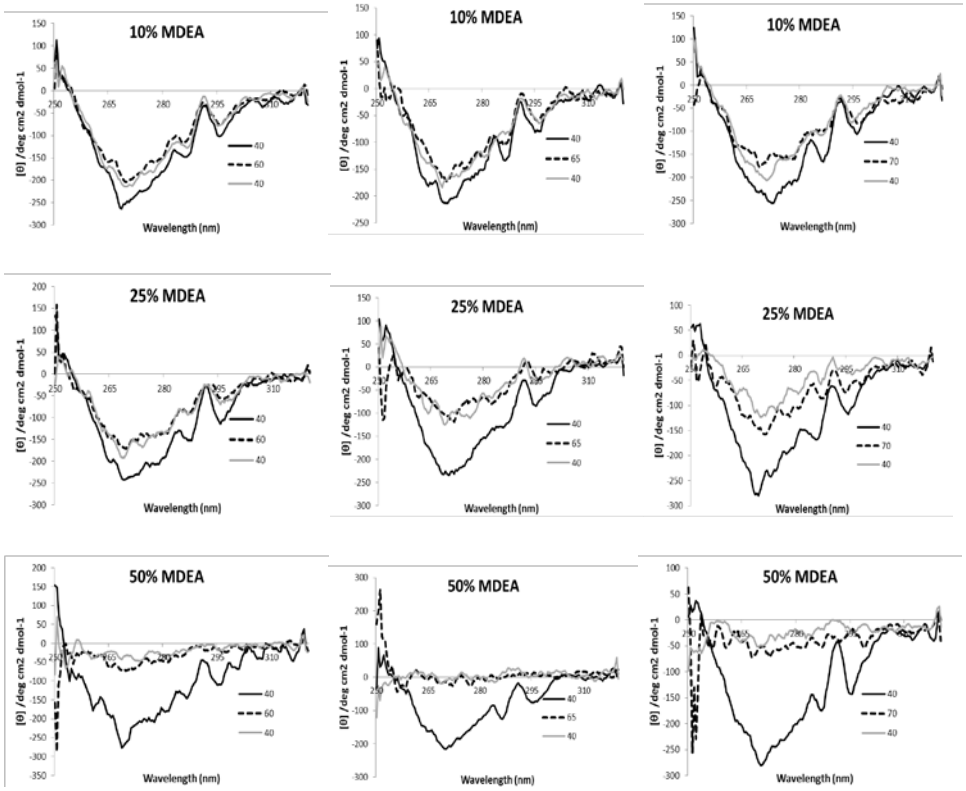


Figure 11 CD-spectra of HCA II_{Eng3} in 2, 5, 10, 25, and 50 % MDEA (top to bottom). Initial temperature in all experiments was 40 °C followed by temperature jumps to 60 °C (left column), 65 °C (middle column), and 70 °C (right column). First 40 °C measurement is seen as the solid black line, maximum temperature as a broken black line, and spectra at subsequent 40 °C after cooling as solid grey line.

Under the conditions used in this experiment it is evident the addition of MDEA, independent of concentration, has a very limited effect on the folded structure of HCA II_{Eng3} at 40 °C after incubation over 10 minutes. On the other hand, when the temperature is increased the presence of MDEA at high concentrations, *i.e*. 25 and 50 %, either denatures the protein fully or induces structural changes that are irreversible.

In the presence of 5 and 10 % MDEA the protein maintain its structure when the temperature jump is between 40 and 60 °C, whereas it is able to refold the slight loss of structure induced by increasing temperature to 65 °C. The induced loss of structure, albeit a small loss, by increasing temperature to 70 °C is however irreversible. The presence of 2 % MDEA has no effect on the structure independent of the temperature maximum. Although high concentrations of MDEA is evidently not possible to use, these results are still promising considering that when the biogas purification process is performed using 50 % MDEA, to drive the capture of CO_2, the level of CO_2 saturation of the MDEA is only 20 %. Thus, by adding HAC II_{Eng3} to drive the conversion of CO_2 to H^+ and HCO_3^- it might still be possible to use 5 – 10 % MDEA to achieve the same level of capacity as an uncatalyzed process, but still with a higher efficiency. Which would then have effects on both environmental and energetic/economic aspects of the process.

With the information about how HCA II_{Eng3} behaves in the presence of MDEA within a rather large concentration span, we decided to narrow the concentration span to around 10 % MDEA. Looking at the stability of HCA II_{Eng3} in this range would give insight into how stable the enzyme would be in concentrations of MDEA where the

capacity of 10 % base would be the same as what it is at 50 % in the absence of enzyme, i.e. assuming that addition of HCA II_{Eng3} can drive the conversion of CO_2 to $H^+ + HCO_3^-$ so that the saturation level of MDEA is close to 100 %.

The thermal stability of HCA II_{Eng3} was measured using DSC and the enzyme was mixed with 0, 2, 5, 8, 10, 12, and 14 % MDEA (Fig. 12). This showed that, in this concentration range, the melting point of the enzyme reaches a plateau at 8 % MDEA that is sustained until 14 %, and possibly higher. T_m in this range is 67.0, 67.0, 66.9, and 66.7 °C, for 8, 10, 12, and 14 % MDEA. These results further indicates that the enzyme can be utilized in the biogas purification process at these concentration, and that this can be achieved with lowered amounts of MDEA. However, the pressure over the desorption column would need to be lowered to approx. 200 mbar to reach a boiling point of below 60 °C.

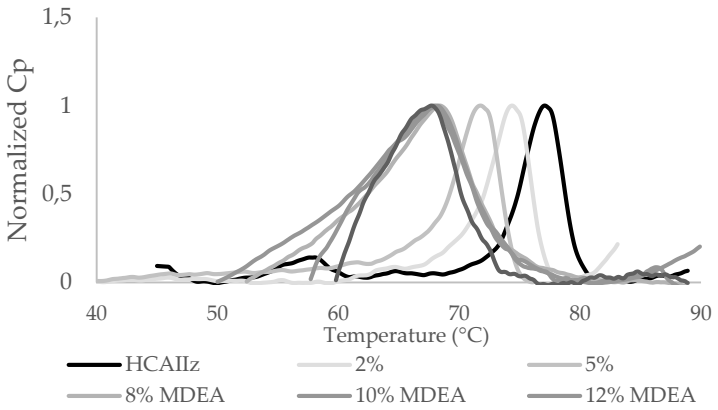


Figure 12 Thermograms showing the specific heat as a function of temperature of 500 µg HCA II_{Eng3} in buffer, 2, 5, 8, 10, 12, and 14% MDEA.

3.1.3 Stability of HCA II_{Eng3} in the presence of TEA

Although MDEA is a good proton acceptor fully miscible with water, it has a drawback when it comes to interacting with proteins. Proteins tend to behave poorly in solutions less polar than water, and MDEA contains a methyl side-chain that gives the molecule a slight hydrophobic character (Figure 13), which at 50 % mixture is likely to influence the polarity of the amine/water mixture. It was therefore decided to investigate whether this hydrophobic side-chain affected the thermal stability of the enzyme and whether it would be possible to replace MDEA with a more hydrophilic base. For this investigation triethanol amine (TEA) was used as a model amine, which instead of a methyl group has an additional hydroxyl group making it more polar (Fig. 13). DSC measurements were therefore also performed with HCA II_{Eng3} in solutions containing TEA instead of MDEA.

Figure 13 Chemical structures of MDEA (left) and TEA (right).

Thermal unfolding of HCA II_{Eng3} in the presence of TEA was measured at 30 °C to 90 °C. For clarity, data in figure 14 is shown between 62 to 87 °C. These measurements clearly shows that the differences in hydrophobicity of the bases MDEA and TEA has a

large effect on the thermal stability of the enzyme. At 10 % TEA, the T_m of HCA II_{Eng3} had decreased to 73.1 °C as compared to the T_m 77.6 °C when the enzyme is in a buffered water solution, but this is only a loss of 4.5 °C compared to 9.5 °C in MDEA at the same amine concentration.

Further, by increasing the amount of TEA up to 20 % it is evident that the change in thermal stability of HCA II_{Eng3} is low. At 20 % TEA the T_m has only decreased to 70.1 °C, which can be compared to the T_m of HCA II_{Eng3} in 25 % MDEA at 62.1 °C, which is 8 °C lower. But it should also be noted that in MDEA the melting curve is broadened and the melting starts somewhere below 40 °C (Figure 10). As is the case with the thermal stability of HCA II_{Eng3} in MDEA at concentrations of 8 – 14 %, the melting point of HCA II_{Eng3} in solutions containing TEA reaches a plateau at 16 % that lasts until 20 %. T_m for the concentrations of TEA are 73.1, 72.2, 70.8, 70.8, 70.5, and 70.1 for 10, 12, 14, 16, 18, and 20 % TEA, respectively.

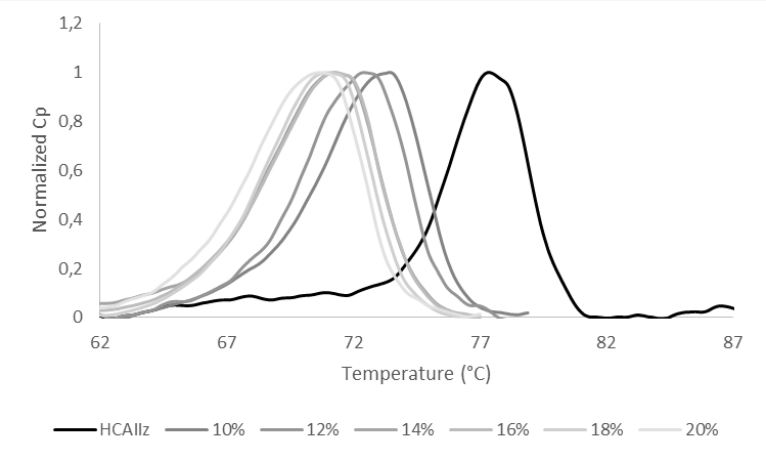


Figure 14 Thermograms showing the specific heat as a function of temperature of 500 µg HCA II_{Eng3} in buffer, 10, 12, 14, 16, 18, and 20 % TEA.

These results opens up the possibility to use TEA instead of MDEA in the biogas purification process since the boiling point of aqueous solutions of TEA is approx. the same as pure water up to 30 % TEA. However, pure TEA and MDEA, although similar has a number of differences in their physico-chemical properties. The one main difference between the two, apart from polarity, is the viscosity. Pure TEA has a viscosity that is 8 times higher than that of pure MDEA, and the absolute viscosity of a 20 % aqueous TEA solution is 1.5 Centipoise (Cp) at 30 °C, as compared to pure water with an absolute viscosity of 0.75 Cp at the same temperature.

Thus, the trade-off for an amine solution that promotes higher stability of the enzyme could be a higher viscosity. This higher viscosity of aqueous solutions of TEA could therefore affect the performance of the absorption/desorption unit by influencing the flow/wetting properties of the liquid. It is however very hard to predict what is the best compromise, a relatively low concentration/capacity of MDEA or a relatively high concentration/capacity of TEA. This and many more effects such as from e.g. different surface tensions of liquids would need to be tested in a laboratory scale unit.

3.1.4 Stability of HCA IIEng3 in the presence of K_2CO_3

From the above information it is obvious that HCA II is sensitive to the polarity of the medium. We therefore also tested the thermal stability of HCA II_{Eng3} in 20 % (w/v) K_2CO_3, which has been suggested for CCS, either by itself or in mixed salts [41, 42]. As is evident in fig. 15 this solution *increases* the thermal stability, even one compared to HCA II_{Eng3} measured in phosphate buffer. From this it can be concluded that the lowered stability in MDEA or TEA is unlikely a simple pH effect, since 10 mM solutions of K_2CO_3 and above have a pH of 11, and is more likely to be due the lower polarity of MDEA and TEA. Proteins can be both stabilized or destabilized depending on protein, types of salt, salt concentration, temperature etc. In this case, for this protein, addition of high concentrations of K_2CO_3 is apparently stabilizing, which is a good indication for further studies using K_2CO_3 as a CO_2 solvent in an enzyme catalyzed process. However, it has also to be kept in mind that any hydrophobic interactions will be reinforced which may cause "salting-out" or stronger protein/surface interactions causing surface induced denaturation.

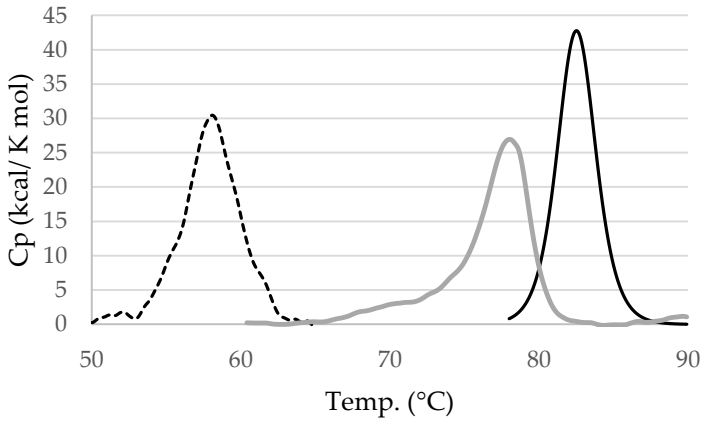


Figure 15 Thermogram over HCA II_{Eng3} in 20 % (w/v) K_2CO_3, pH > 11 (solid black, T_m 82.7 °C), as compared to HCA II_{pwt} (dotted black) and HCA II_{Eng3} (grey) in 10 mM phosphate buffer, pH 7.5.

3.2 FURTHER DEVELOPMENT OF THERMALLY STABLE HCA II VARIANTS

Even though the enzyme variant HCA II_{Eng3} is a highly stable variant of the highly active and efficient Human carbonic anhydrase II, it can from the results be understood that additional physical stability would be beneficial, preferably also strengthening the kinetic stability. We therefore used computational methods (SSBOND), and primary structure analysis of related α carbonic anhydrases to determine locations within HCAII were other disulfide bridges could be incorporated (Fig. 16).

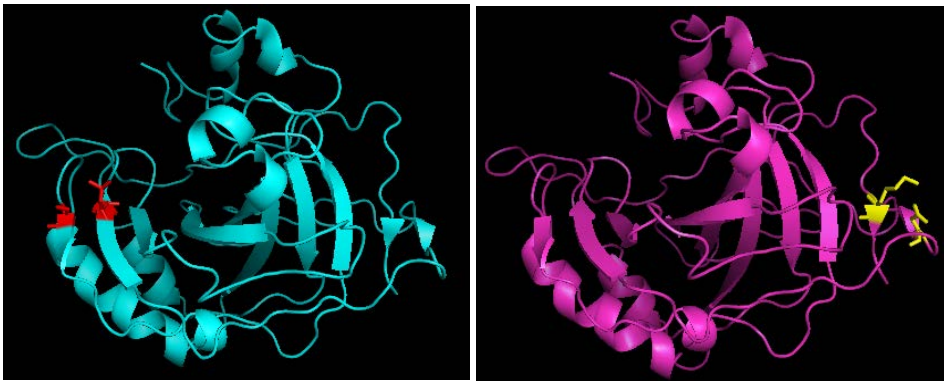


Figure 16 3D structures of HCAII which show the position where the newly designed disulfide bridges are inserted. Left: location of the disulfide between L60C/S173C (highlighted red). Right: location of the disulfide between D41C/K257C (highlighted yellow).

Two such possible locations were identified, one that could form a disulfide bridge if L60 and S173 were replaced by cysteines, and one that could form a bridge between D41 and K257 and actually close an N-terminal knot of the enzyme. These are also situated in positions believed to form late in the folding of the protein and could therefore also, if formed, add to the kinetic stability of the protein.

Site-directed mutagenesis were employed to insert the cysteines into the sequence of HCA II_{pwt}. Successful site-directed mutagenesis was confirmed by DNA sequencing, and the vector was transformed into *E. coli* BL-21/DE3 cells and expressed and purified as earlier described. The purified enzymes were analyzed for detection of free cysteines, and it were found that both the disulfide bridges formed spontaneously during expression. CD-spectra of the two mutants were then collected to determine that they had folded correctly during expression, as well as to test their thermal stability. Data were collected between 250 – 320 nm at 40, 70, and then 40 °C again (Fig. 17).

These measurements showed two interesting results. Firstly, in fig. 17 A, it can be noted that the variant HCA II_{Eng5} (D41C/K257C) lost most of its structure upon heating, but unlike any other known variants of HCA II, it regained all of this structure when the sample was cooled down to 40 °C again! Secondly, in fig. 17 B, it indicated that the mutant HCA II_{Eng4} (L60C/S173C) has a high T_m, since it had a preserved structure even at 70 °C.

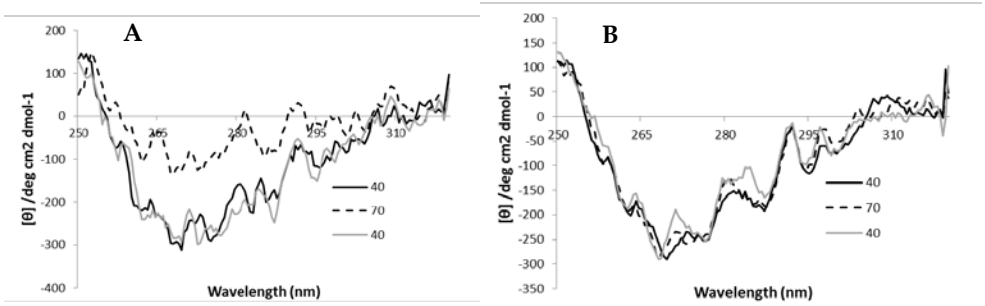


Figure 17 CD-spectra of novel HCA II variants. (A): HCA II_{Eng5} and (B): HCA II_{Eng4} collected in 20 mM sodium phosphate buffer, pH 7.2. Spectrum after 10 minutes incubation at 40 °C (black line), after 10 minutes incubation at 70°C (dashed black line) and after subsequent cooling and incubation at 40 °C (solid grey line).

To further investigate the thermal stability of the two variants their thermal unfolding was determined using DSC. The temperature scanning of HCA II_{Eng5} showed that the insertion of the disulfide bridge between D41C and K257C indeed did increase the T_m of the variant, although the increase is only 3.6 °C (T_m = 62.1 °C). The low increase in T_m was however expected since in this case two charged amino acids, the negatively charged aspartic acid (D) and the positively charged lysine (K) were exchanged cysteine and it is very likely that these two are part of a larger electrostatic network on the surface of the enzyme, in the folded state. The main reason for testing this variant was because the disulfide bridge would, if successful, bind together the very C-terminal part of the enzyme (amino acid no. 257 out of 259) and more central parts of the enzymes. The formation of this C-terminal knot is known to be one of the final events in the folding of the enzyme and therefore a good candidate for increasing kinetic stability if the enzyme were still able to fold.

In fact, the somewhat contradictory results between the CD experiment, in which the enzyme were not fully denatured after 10 min of incubation at 70 °C (fig. 17 A), and the determined T_m (fig. 18), could indicate that the variant has an increased kinetic stability, so that 10 minutes at 70 °C is not enough to fully denature the enzyme in that time. This very interesting feature and the enzymes apparent ability to fully refold after heating to 70 °C suggests more studies of this variant to determine structure, activation energy for unfolding, unfolding kinetics structure of unfolded state etc.

The temperature scanning of HCA II_{Eng4} gave however rise to two melting events, one at 58.3 °C and one at 77.3 °C (Fig. 18). The T_m at 58.3 °C initially lead us to believe that the sample had been contaminated with HCA II_{pwt}, although it could also be the case that the disulfide bridge could form in two different conformations, one that is highly stabilizing and one that in principle has no effect on the stability.

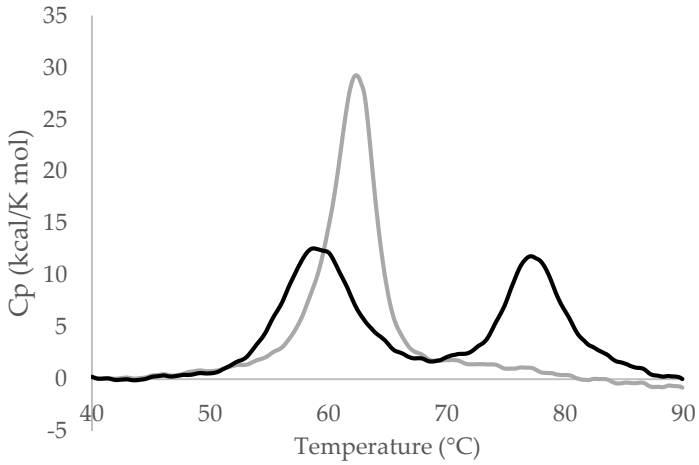


Figure 18 Thermograms showing the specific heat as a function of temperature of 500 µg HCA II_{Eng5} (grey line) and HCA II_{Eng4} (black line) in 20 mM sodium phosphate buffer, pH 7.5.

In order to determine which one of these possibilities was correct, the enzyme were treated with a redox buffer. One aliquot of the sample was treated at room temperature and another aliquot at 37 °C in presence of a mixture of oxidized and reduced dithiotheitol (DTT) in a ratio of 1 mM/3 mM, both overnight. The thermal stability of the two samples were then determined by DSC (after removal of the redox reagent). This showed that the sample that had been treated with redox reagent at room temperature had the same thermal melting behavior as the variant had after expression

(Fig. 19). However, the sample that had been treated with the redox reagent at elevated temperatures showed a single peak at 76.7 °C, indicating that the bridge between L60C and S173C can form in two conformations during expression but that the more thermally stable bridge forms under oxidative conditions at higher temperatures.

It is also worth noticing that the introduction of the single L60C/S173C disulfide bridge has a very large effect on the thermal stability for a single disulfide bridge, as is seen in Fig. 19 (bottom graph). This single bridge in fact increases the T_m of the enzyme to the same thermal stability as is reached by the two bridges in HCA II_{Eng3}, *i.e.* the single bridge L60C/S173C increases the T_m by 18.1 °C.

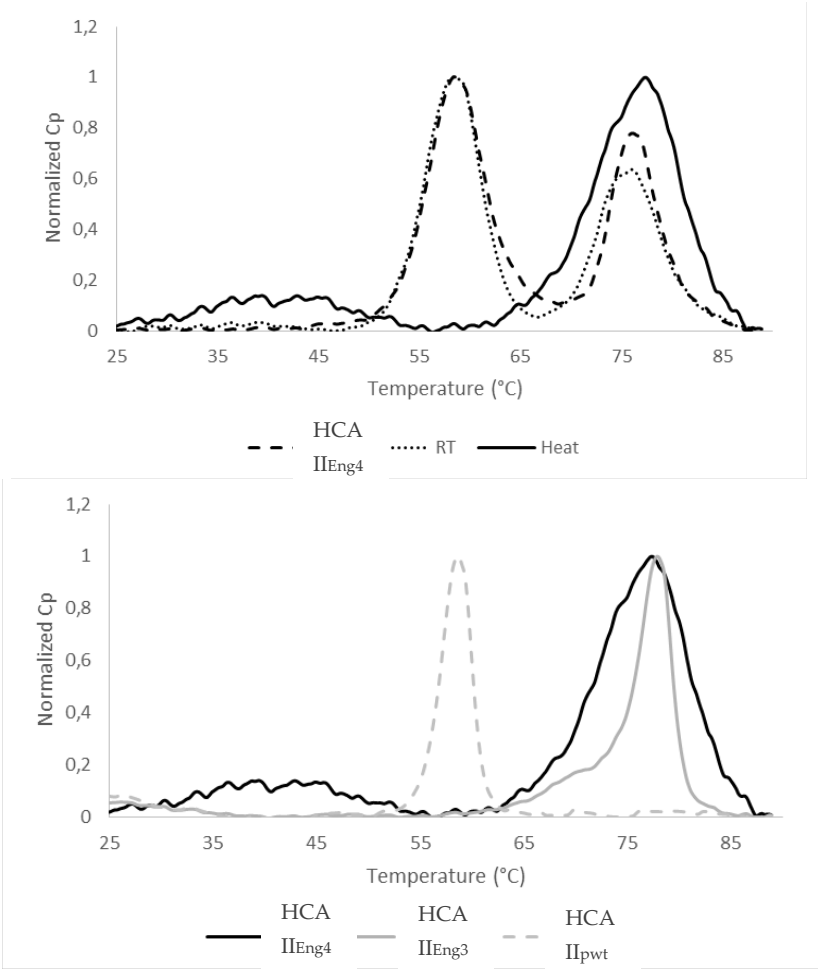


Figure 19 Thermograms showing the normalized specific heat as a function of temperature of 500 µg HCA II_{Eng4}. Top: after expression (dashed black line), after redox reaction at room temperature (dotted black line), and after redox reaction at 37 °C (solid black line). Bottom: HCA II_{pwt} (dashed grey line), HCA II_{Eng3} (solid grey line, and HCA II_{Eng4} (solid black line) in 20 mM sodium phosphate buffer, pH 7.5.

We then proceeded to construct a mutant which could potentially have an even greater thermal stability, by introducing the disulfide L60C/S173C into the mutant HCA II_{Eng3}. Once again site-directed mutagenesis was employed, followed by expression and purification. Thermal stability studies on the newly expressed enzyme, HCA II_{Eng6} showed that, as was the case with HCA II_{Eng4}, that the enzyme melting curve was more complex. HCA II_{Eng6} showed three melting events (Fig. 20 grey line).

Thus, the introduction of yet another pair of cysteines complicates folding as there are more cysteines that, if they are not spontaneously formed correctly, need to find each other in order for the enzyme to adopt the most stable form. The major melting event of

HCA II_{Eng6} however takes place at 83.6 °C, an increase of 24.6 and 5.6 °C as compared to HCA II_{pwt} and HCA II_{Eng3}, respectively. There are two smaller melting peaks, one at around 68.1 °C which most probably corresponds to a portion of the enzyme that has only formed a single disulfide (most probably between S99C and V242C), and there is one peak at 56.8 °C which might correspond to a portion of the enzyme where no disulfides had formed.

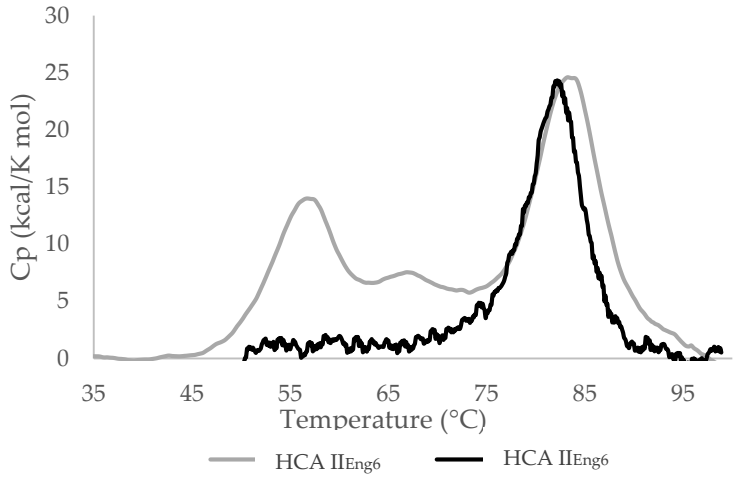


Figure 20 Thermograms showing the specific heat as a function of temperature of 500 µg HCA II_{Eng6} after expression (grey line) and after redox reaction at 37 °C (black line).

An overnight incubation at 37 °C in the presence of redox reaction reagent made sure that all three disulfides formed according to the design (S99C/V242C, A23C/L203C, and L60C/S173C). The correctly oxidized HCA II_{Eng6} had a T_m of 84.1 °C. Thus, in this case the thermal stability was not cumulative, in which case the Tm would have reached 102.9 °C. This is probably so because strains are introduced in the structure when all three disulfide bridges are present. This mutant is also more stable than the carbonic anhydrases of *Methanosarcina thermophile* (Mt-CamH) with a T_m of 70 °C and *Methanobacterium thermoautotrophicum* (Cab) with a T_m of 75 °C, but falls short by roughly 16 °C in comparison to SspCA of *Sulfurihydrogenibium yellowstonense YO3AOP1*.

3.3 PILOT SCALE ENZYME PRODUCTION (MEDICAGO AB)

3.3.1 Protein production

E. coli was cultured in 13 L bioreactors using a feed batch culturing technique in a standard Medicago AB growth medium. The main source of carbon was glycerol a cheap byproduct from FAME (fatty acid methyl ester, biodiesel) production. The expression of protein was induced with IPTG. Each batch produced >10 g HCA II_{Eng3} per liter medium.

3.3.2 Protein purification

An affinity gel were synthesized based on the affinity gel used at InZymes Biotech AB. A benzensulfonamide ligand was coupled to an agarose gel via an epoxy group. The purification step of the enzyme was modified, compared to what is used at InZymes

Biotech AB, by replacing the sodium azide used for elution with the non-toxic sodium perchlorate combined with a lowered pH which proved to work well. Thereby avoiding the use of the poisonous and carcinogenic sodium azide.

3.4 DESIGN OF A LABORATORY SCALE GAS TREATMENT UNIT

Based on all the above and Purac Puregas ABs experiences of the CApure process, a laboratory scale absorption/desorption unit suitable for testing an enzyme based process as described was designed in discussions between Purac Puregas AB, InZymes Biotech AB and SAXE Nordic AB/Normag Labor- und Prozesstechnik GmbH (Figure 21).

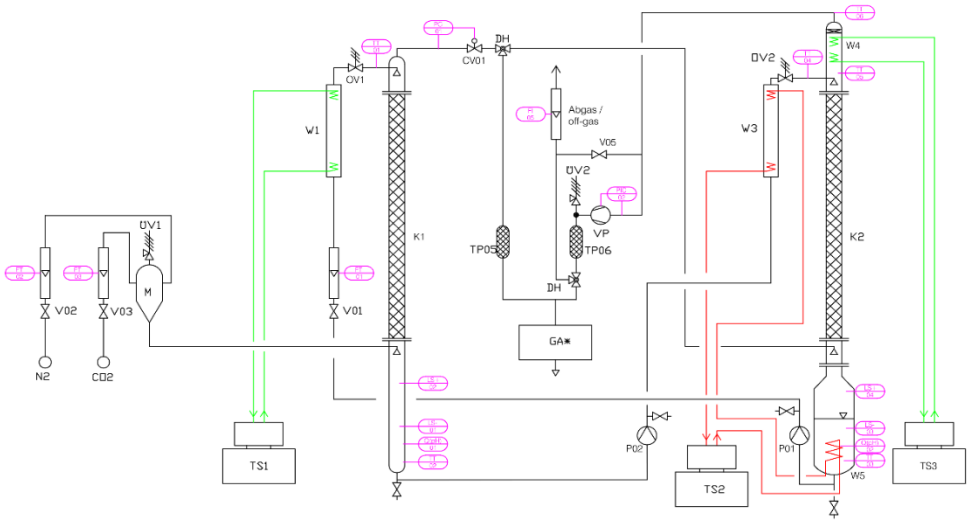


Figure 21 Blue print of laboratory scale amine gas treatment unit*.

This process would, simplified, have included:

- Valves (V02/V03), gas rotameters (FT 02-03) and mixer (M) for CO_2/N_2
- K1: absorption column with packing material and a top liquid distributor
- TS1 and W1: cooling thermostate and liquid feed cooler for K1
- K2: desorption column with packing material, top liquid distributor, demister and including buffer volume for absorption media and immersion heater (W5)
- W4: condensation unit for K2,
- W5: immersion heater
- TS3: cooling thermostate for W4
- W3: liquid feed heater for K2
- TS2: heating thermostate for W3/W5
- P01/02: Recirculation pumps
- VP: Vacuum pump that automatically adjusts process pressure from vapor pressure
- GA: gas analyzer
- The additional level switches, valves, instrumentation (flow-, temperature- and pH meters) and data logging for testing and process development.

**Blueprint is the property of, and included by the approval of, SAXE Nordic AB/Normag Labor- und Prozesstechnik GmbH.*

4 Concluding remarks

In this project we have investigated the prerequisites for designing an energy efficient and enzyme catalyzed CO_2 capturing process for biogas upgrading. Unfortunately, with respect to the process itself, it had to stop at the literature studies and theoretical reasoning. Nevertheless, based on Purac Puregas experiences of the CApure process and what was found regarding the engineered enzyme variants properties and behavior in various CO_2 capturing solvents, we believe that the proposed process would have been a good starting point if it could have been realized.

Moreover, even over the duration of the project, there has been a rapid development of solvents for CCS. However, some of these will be more suitable for uncatalyzed processes, whereas other will be more suitable for enzyme catalyzed processes, depending on their influences on the enzyme activity, stability etc. It is important to understand the difference!

What can also be concluded is that for an enzyme catalyzed process, the conditions and solvents need to be adjusted to suit the enzyme, or it will *not* be an enzyme catalyzed process for very long. However, it is apparent from even these relatively few measurements that there are a lot of parameters to play around with to reach a process with conditions that are likely to work. The other way round, to fit the enzyme to the process, is of course also an alternative. For a real life functional and economical process this will most likely go far beyond the simple thermal stability of the enzyme and includes production costs, lifetime limitations (for whatever reason), immobilization cost (if needed) and cost to replenish the enzymes of the process. Although not analyzed in an actual process, the herein examined enzymes could contribute to the work of designing such a feasible process. It is further possible that by combining some of the analyzed variants, novel enzymes with even higher thermal, thermodynamic and kinetic stability could be combined, maybe also including better refolding capacity!?

From the high degree of activity in the field it would appear that most problems to reach functional processes should be solved in the near future. However, whether these will be implemented or not will depend on the operational costs based on choice of solvents/enzymes and the process energy demand. Therefore it would appear "meaningless" to adapt enzymes etc. to processes with regeneration temperatures above 100 °C, since these process are likely to be too energy intense, regardless if the use is for CCS or for upgrading of biogas. Thus, the way forward seem to be low energy solutions, in which case also the enzyme thermostability issues would be less of a problem. That is, a process design close to the one presented in this report.

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ENZYMATIC UPGRADING OF BIOGAS

Den högsta förädlingsgraden av biogas är i form av fordonsgas. Nuvarande uppgraderingsmetoder har låg skalbarhet och är energiintensiva då de baseras på höga tryck eller höga temperaturer. På grund av dessa faktorer begränsas därför fordongasproduktionens utbyggnad av att det inte finns mer driftsekonomiska och mer skalbara uppgraderingstekniker.

Utvecklingen inom koldioxidinfångning och lagring (Carbon Capture and Sequestering, CCS) utvecklas snabbt mot mer energieffektiva metoder, varav ett utvecklingsspår är att använda enzymer för att öka effektiviteten hos lösningsmedel som har hög koldioxidbindande kapacitet, men ändå kräver låg regenereringsenergi. I princip skulle kunskapen från detta område vara applicerbart för att utveckla också väsentligen mer energieffektiva uppgraderingstekniker för biogas.

Denna rapport innehåller en genomgång av de principiella funktionerna för olika lösningsmedel för enzymbaserad koldioxidinfångning, aktuella processer samt enzymer. Utifrån detta och tillgängliga enzymers egenskaper presenteras också en teoretiskt möjlig, skalbar och energieffektiv processlösning.

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