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We devised an enzyme-based facilitated transport membrane bioreactor system to selectively remove carbon dioxide (CO2) from the space station environment. We developed and expressed site-directed enzyme mutants for CO2 capture. Enzyme kinetics showed the mutants to be almost identical to the wild type save at higher pH. Both native enzyme and mutant enzymes were immobilized to different supports including nylons, glasses, sepharose, methacrylate, titanium and nickel. Mutant enzyme could be attached and removed from metal ligand supports and the supports reused at least five times. Membrane systems were constructed to test CO2 selectivity. These included proteic membranes, thin liquid films and enzyme-immobilized teflon membranes. Selectivity ratios of more than 200:1 were obtained for CO2 versus oxygen with CO2 at 0.1%. The data indicate that a membrane based bioreactor can be constructed which could bring CO2 levels close to Earth

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GOAL

The goal of this project was to attempt to develop an enzyme-based bioreactor to selectively extract carbon dioxide from respiratory gases with reference to the space station environment and possible further application to the space suit.

OBJECTIVES

Key objectives were: to develop an enzyme-based facilitated transport bioreactor to remove CO_2 from respiratory gases, to develop a system which was regenerable on-orbit thereby reducing mass, and to develop a system which would be able to achieve an ambient carbon dioxide level lower than that possible by other existing methods. The system had to be small and light.

APPROACH

To achieve these objectives we elected to use a biomimetic approach wherein enzyme would be used as the capture facilitating element. The system would be designed to operate under normal pressure and temperature.

SPECIFIC AIMS

The goals and objectives would be satisfied if five Specific Aims were realized:

1) to recombinantly engineer the enzyme and develop a DNA expression system for it using *E. coli*,
2) to immobilize the enzyme to a carrier,
3) to construct a bioreactor to selectively extract CO_2 from respiratory gases,
4) to develop a system for easy regeneration of the immobilized enzyme, and
5) to study the CO2-reactor systems and logistics impacts assessing safety, reliability and maintainability.

Drs. McGregor, Willson and Rudolph participated in all stages of this project.

Our first effort was to carry out a detailed analysis and prioritization of the several options presented in the originating proposal to determine which experimental path held the greatest likelihood to satisfy the stated goals and objectives. This was necessary for two reasons. First, we had proposed several alternative paths to achieve the same objectives and it was necessary to choose among these. Second, a number of important developments had occurred during the grant evaluation period in the areas of immobilization chemistry and in the molecular biology of the key enzyme, carbonic anhydrase (CA) which necessitated consideration. Most importantly, over this time several isozymes of the enzyme under investigation were cloned. These data supported our resolve to use cloned and genetically modified enzyme. Dr. Philip J. Laipis joined our group to provide access to the CA clones and the *E. Coli* expression system.

ENZYME RECOMBINANT ENGINEERING AND EXPRESSION

We had proposed that a preferred path to regenerability was to maintain the shell of the bioreactor in place and to remove and replace the core catalytic material as warranted. To accomplish this we determined that it would be best to engineer a controllable linker between the enzyme and the immobilization surface. Dr. Klibanov participated in the design and immobilization concept generation. To achieve this end we began a series of experiments in site-directed mutation with the expectation that we would be able to express the mutants satisfactorily in *E. coli*. In collaboration with Dr. Laipis we set to mutate the CA2 clone by the addition of a polyhistidine (His) tail at each the amino or the carboxy end of the enzyme. This His tail would be used later to facilitate immobilization to a metal ligand.

We constructed a human carbonic anhydrase type 2 [HCA2] expression vector suitable for inserting a six histidine peptide [His-tag] and carried out the steps required for insertion of the His-tag oligonucleotide sequence. To insert the His-tag we rearranged the HCA2 expression vector by removing a restriction site and inverting the f1 ORI normally used for mutagenesis.

Five genetically altered HCA2 clones were generated which inserted a six histidine oligonucleotide (His-tag residue) at each the amino or the carboxy termini of the enzyme. Restriction enzyme analysis and DNA sequencing of the resulting clones revealed that four of the five were correct, with a His-tag in the correct orientation to allow further use.

Initial assessment of total activity revealed that the amino terminal His-tag recombinant enzyme exhibited activity (R1 reaction - the hydroxylation of CO_2) almost identical with the wild type, unaltered enzyme. In contrast, the carboxy terminus His-tagged HCA2 clones showed reduced activity (approx. 5-fold). However, by selecting additional colonies from the initial isolates, several were obtained where the specific activity of the carboxy-terminus clones was comparable to that of the amino terminal-tagged sample. Six liter cultures were then grown for both the amino and carboxy terminal-tagged clones. These showed high total activity, and a specific activity comparable to the unmodified HCA2. Purity of the product is high, >90% on the basis of SDS gel electrophoresis. Both His-tag variants showed an appreciable yield (43-48 mg).

Preliminary kinetic studies, using ^{18}O to measure enzymatic activity indicated considerable similarities in details of the kinetic properties of both the amino and carboxy terminus His-tag clones and the wild type enzyme. However, addition of the His-tag resulted in some mild perturbations of Kcat and an elevation in activity in the pH titration curve at higher pH as compared with the wild type. Thus, the second key reaction (RH_2O) the hydrolysis of water to provide reactive hydroxyl groups was diminished. The significance of this alteration would await immobilizations studies. This work was carried out in conjunction with Drs. Tu and Silverman.

ENZYME IMMOBILIZATION

The second aim was to immobilize the native enzyme to a carrier. To attain this aim we carried out investigations of various immobilization methods as applied to a variety of immobilization surfaces. These studies were done in conjunction with Drs. John F. Kennedy, Marion Paterson and Daniel Thomas and Mr. Robert Landers.

Using native enzyme, we tested several different immobilization support surfaces and immobilization chemistries. These included 3 forms of nylon, methacrylate beads, Ni-NTA sepharose beads and three different types of glass surfaces. The glass was activated by silanization and, in some cases, binding of titanium chloride to provide another type of metal ligand. Initial analyses suggested that the Ni-NTA sepharose provided the most efficient binding, with values 3 to 5.5 times greater than with other supports. However, inasmuch as most of the immobilized enzyme was in the interstices of the sepharose beads mass transfer limitations reduced the retained activity (RA). In contrast, nylon polyethyleneimine immobilized enzyme and epoxy activated methacrylate beads were about one-third as active. The RA using nylon polyethyleneimine was 27%. Using spherosil glass beads the RA was 17% for and it was somewhat lower for other types of glass.

We investigated the Ni-NTA binding using an immobilization surface superior to Ni-NTA sepharose, namely Ni-NTA silica. Both the carboxy-His-6 tailed CA and the amino-His-6 tailed HCA2 were immobilized to Ni-NTA silica. Each exhibited RA in excess of 12%. Both were resistant to repeated washing with buffer. However, they could be removed from the chelated metal immobilization site by the competitive agent imidazole. The eluted His-6 HCA2 exhibited full activity in bulk solution indicating that sole element of the immobilization reduced its activity. The Ni-NTA was available for a next round of immobilization. The attachment-removal-reattachment sequence could be repeated at least five times. This observation is important to allow on-orbit regeneration of the bioreactor by means of only a few grams of enzyme.

We carried out a mass transfer analysis of these various immobilization regimens. The results suggested that intermolecular distance, surface effects, flow limitations in bead interstices and enzyme orientation are all important parameters in evaluating efficacy of such bioreactors. The consequence of this analysis was that a future goal would be to space the enzyme catalysts sufficiently far from one another and from the surface to maximize activity while minimizing enzyme used.

MEMBRANE BIOREACTORS

Our third aim was to demonstrate proof-of-concept by creating a membrane with selectivity for carbon dioxide (CO_2) to extract CO_2 from respiratory gases. The ability to remove CO_2 at partial pressures below 0.3% was used as an efficacy criterion. Drs. Tu, Silverman and Thomas and Mr. Landers participated in this aspect of the work.

We pursued this aim in several different ways. In the first, we created proteic films as selective high flux membranes for gas transfer. Proteic films are made by cross-linking the enzyme of choice alone of with the addition of one or more additional proteins to make a hydrogel-like material. Bifunctional agents such as dialdehydes are typical cross-linkers with glutaraldehyde being used most commonly. Albumin is commonly used to increase the structural stability of the gel.

Proof-of-concept results using early design proteic membranes, measured by mass spectrometry, showed as much as a five-fold improvement in rate of CO_2 capture over control membranes. Maximal improvement was seen at 1% CO_2 at 1 ATM. At 0.1% CO_2 the membrane exhibited a 76% improvement over controls. At 0.3% CO_2 the improvement is about 3-fold. Calculations indicate that an ideal CA-based proteic membrane could exhibit selectivity for CO_2 over oxygen and nitrogen in excess of 2 orders of magnitude.

In the second we created thin liquid film membranes by trapping a buffer-CA solution in the interstices of Anodisc membranes. Anodiscs are alumina membranes with uniform columnar pores of 0.1 μm in diameter. They are 60 μm thick and can be filled with 800 μl of fluid. They act like ultimate rubbery membranes with high solubility for CO_2 and diffusivity values for ions and gases within an order of magnitude of one another. Due to the increased solubility the alpha value, i.e., relative permeability ($P_{CO2/O2}$) favors CO_2 by many-fold. The implication of these studies is that a CA-based proteic or facilitated transport membrane should achieve CO_2 partial pressures of 0.05% under CELSS conditions.

In a third approach we immobilized bovine CA2 to highly permeable teflon membranes via a standoff made of poly(vinyl alcohol) hydrogel. Each of these membranes showed significant facilitation of CO_2 transport. Selectivity factors of CO_2:O_2 of 206-fold were seen at 0.1% CO_2, 74-fold at 1% CO_2 and 26% at 10% CO_2. The membranes were progressively more efficient as the CO_2 partial pressure decreased. This result suggests that we should be able to achieve near ambient CO_2 levels for the crew. Initial data show the teflon membranes to be resistant to fouling

SYSTEM LONGEVITY AND REGENERATION OF IMMOBILIZED ENZYME

LONGEVITY

Lyophilized enzyme has a shelf life on the order of tens of years. Similarly, even simple dried enzyme appears to last for surprisingly long times. We have demonstrated the enzyme in aqueous solution, held at 4°, retains complete viability for over 30 days. (In year three we continued this avenue of exploration and demonstrated lifetimes at 4° for over 2 years with no loss of activity).

REGENERATION

Either of these storage methods, dry or at 4°, when used in conjunction with the His-6 tailed enzyme immobilized to a Ni-NTA surface would allow regeneration of the membrane system for five times and could cover a span of at least two years.