

/Identification and Characterization of a Succinate  
Receptor Protein on Rhizobium trifolii 0403./

by

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## LITERATURE REVIEW

Bacteria in the genus Rhizobium are capable of forming symbiotic relationships with their respective plant counterparts. These bacteria, which are commonly found in the soil, may be in close proximity to the roots hairs of their symbiotic partner. The bacteria metabolize tryptophan given off by the root cells into indolacetic acid which acts as a plant hormone and causes the root hairs to begin to curl around the bacteria. The bacteria are then capable of penetrating the root hair cells and inducing formation of an infection thread. Many cells in the plant cortex are penetrated by the infection thread, become infected, and are stimulated to divide resulting in a capsule, or nodule, containing the Rhizobium cells.

Once the Rhizobium cells find themselves within the environment of the nodule, they undergo very complex changes. They lose the ability to divide and become bacteroids--rod shaped cells which are swollen on one end or in the center. It is in this state that these organisms are capable of converting atmospheric nitrogen into ammonia.

There is considerable evidence that the tricarboxylic acid intermediates, especially succinate, play a crucial role in nitrogen fixation by organisms in the genus Rhizobium. Utilization of tricarboxylic acid intermediates has been shown to be related to symbiotic effectiveness in a variety of species (1,19,5,23,39,40). It has also been shown that succinate added to an exponentially growing culture of Rhizobium trifolii induces swelling and the pleomorphic shapes characteristic of nodule bacteroids (46,44). Furthermore, it has also been shown that nitrogen-fixing activity ( $^{15}\text{N}$  incorporation and acetylene reduction) accompanies in vitro bacteroid formation (45).

The tricarboxylic acid cycle has been implicated as a central pathway for carbon metabolism in bacteroids (43). Mutants defective in enzymes of this cycle, such as succinate dehydrogenase and alpha-ketoglutarate dehydrogenase, form ineffective nodules (16,22). Interestingly, mutants deficient in carbohydrate metabolism generally form effective nodules (40,43). The tricarboxylic acid cycle intermediates succinate, fumarate, and malate are considered the most likely carbon and energy substrates used by bacteroids, since

mutants defective in dicarboxylate transport (dct) grow normally on other carbon sources but are impaired in nitrogen fixation (2,8). These compounds have also been shown to support nitrogen fixation by purified bacteroids (46,5).

It has long been recognized that micro-organisms metabolize many nutrients present in their growth media via functionally specialized permeation mechanisms which possess a high degree of stereospecificity for the substrate being transported (13). The inducible formation of transport systems affecting the uptake of the dicarboxylic acids had been demonstrated in pseudomonads (27,11,4) and in Aerobacter (14,15), however the specificity of the systems was not resolved. Kay and Kornberg in 1969 reported an inducible system specifically involved in the uptake of  $C_4$ -dicarboxylic acids in E. coli (26). Mutants which lacked the system could still metabolize internal  $C_4$ -acids, but failed to grow on these substances as sole carbon sources. The uptake system was shown to be specified by a gene (dct) located on the E. coli genome close to the xly marker.

Dicarboxylic acid transport studies have been carried out using a variety of organisms for the past

several years and a great deal of knowledge has been gained. In 1977, T. C. Y. Lo showed that there is only one dicarboxylic acid transport system present in Escherichia coli K12 (33). He indicated that at least three genes were involved. They were designated cbt, dct A, and dct B. The products of those genes are: a periplasmic binding protein (PBP) specified by cbt, and two membrane integral proteins, SBP 1 and SBP 2, specified by dct B and dct A, respectively. The SBP 1 and SBP 2 proteins lie in close proximity to one another in the membrane and each appears to bridge the membrane. The substrate recognition site of SBP 1 is exposed on the inner surface of the membrane while the SBP 2 recognition site is exposed on the outer surface.

Lo et. al. in 1979 (32) demonstrated that the dicarboxylate transport component located in the outer membrane could be inactivated by two different kinds of nonpenetrating inhibitors, proteases and diazosulfanilic acid. These inhibitors seem to act on the periplasmic dicarboxylate binding protein. They also showed that by adding this protein to inactivated cells or to transport-negative mutants, they could reconstitute the dicarboxylate transport system. These results suggested that the dicarboxylate binding



protein found on the cell surface plays an essential role in the translocation of dicarboxylic acids across the outer membrane.

C<sub>4</sub>-dicarboxylic acid transport in Rhizobium leguminosarum was shown to play a critical role in that organism's ability to form effective nodules and ultimately to fix nitrogen (19). A great deal of evidence seems to indicate that C<sub>4</sub>-dicarboxylic acids produced by the plant are utilized by bacteroids in N<sub>2</sub>-fixing root nodules (5,23,39,40). In order to determine what role succinate, as well as the other C<sub>4</sub>-dicarboxylic acids, plays in the induction of bacteroid formation and the subsequent initiation of nitrogen fixation, succinate uptake and metabolism in the organism must be more precisely defined.

Finan et. al. (19) examined the transport of succinate in an effective streptomycin-resistant strain of Rhizobium leguminosarum. They noted high levels of succinate transport occurred when cells were grown on succinate, fumarate, or malate. In contrast, they discovered that low transport occurred when cells were grown on glucose, sucrose, arabinose, or pyruvate as the sole carbon source. A succinate dehydrogenase mutant was isolated to study the intracellular

accumulation of succinate. It was found that these mutants transported in and accumulated 400 times the external concentration of succinate. It was also found that succinate transport was completely abolished in the presence of metabolic uncouplers and that succinate transport was competitively inhibited by fumarate and malate. The conclusion of this study was that R. leguminosarum possesses a  $C_4$ -dicarboxylic acid transport system which is inducible and mediates the active transport of succinate, fumarate, and malate into the cell.

In rhizobia, the Dct system appears to be a common transport system for succinate, fumarate, and malate. Conflicting data has been presented regarding the the regulation of the Dct system (19,24,35,42). A cluster of four dct genes has been identified in R. leguminosarum (37). Three of these genes appear to encode regulatory elements and one specifies a structural element.

In 1986, Nixon et. al. reported that the ntrB and ntrC proteins of Bradyrhizobium parasponia share homology with a number of other regulatory

proteins (36). They proposed that the regulatory genes comprise a two-component regulatory system that evolved from a common ancestral system which involved the transporting of information regarding the status of the environment by one protein to a second protein which would carry out some transcriptional function depending upon the signal. On the basis of information they presented, Nixon et. al. proposed a model for transduction of environmental signals by the ntrB and ntrC gene products. As suggested for chemoreceptors (29,7,9) the non-conserved N-terminal domains of the ntrB set of proteins perceive environmental signals and transmit them to the conserved cytoplasmic C-terminal portion of the ntrC-set protein, with the modification affecting activation or repression through an allosteric effect on the C-terminal domain(s) of the ntrC-set protein.

Ronson et. al. (38) propose a model in which the DCT B protein acts as a sensor of C<sub>4</sub>-dicarboxylic acids in the immediate environment of the bacterium. Nucleotide sequencing data indicate that the genes involved in dicarboxylic

acid transport (dct genes) are members of the conserved family of two-component regulatory elements that respond to environmental stimuli. A signal is transmitted via the Dct B protein to the DCT D protein which acts as a transcriptional regulator of the dct A gene product, a permease responsible for transporting C<sub>4</sub>-dicarboxylic acids across the membrane.

In Rhizobium species, genes required for symbiotic nitrogen fixation are located on two megaplasmids (3,18,25,41) and on the chromosome (21,31). The nod megaplasmid contains genes required for early stages of nodule formation, nitrogenase structural genes (nif HDK), and other genes necessary for nitrogen fixation (nif and fix) (10,28,34). Genes which are required for the production of exopolysaccharides are found on the exo plasmid. The products of these genes play a role in the formation of infection threads within the developing nodule (17,18).

Elucidation of the pathways of metabolic exchanges between plant and bacteria are essential in the understanding of symbiosis and its regulation. It has been shown by Watson et al.

that a DNA segment containing multiple dct genes is located on the exo megaplasmid of R. meliloti (47). The authors suggest that there may be some fundamental differences between the regulation of transcription of genes required for symbiotic nitrogen fixation located on the chromosome and those located on the exo megaplasmid.

It was our premise in conducting this study, that there is a unique protein which binds succinate on the surface of R. trifolii 0403 and subsequently that event triggers a cascade of transcriptional events which leads to bacteroid formation and nitrogen fixation. It would seem possible that the Dct system may play a significant role in this process since it is an effective and widely conserved system in bacteria. It also seems quite likely since the Dct system is coded for on the exo megaplasmid of R. meliloti and may play a role in some unique regulatory mechanism for that plasmid. All of these factors in addition to the fact that the Dct system acts in response to environmental levels of  $C_4$ -dicarboxylic acids that have been detected in host legumes indicate that the Dct system may play

a critical role in how R.trifolii 0403 is capable of forming bacteroids in vitro and fix nitrogen in response to specific levels of succinate in the media.

The data described in this study identify and characterize a peripheral protein(s) to which succinate binds on the surface of Rhizobium trifolii 0403 . We believe that this is the DCT B protein which Ronson described (38) and that the binding of this protein by a C<sub>4</sub>-dicarboxylic acid or any structurally related molecule triggers the events which lead to bacteroid formation in vitro. We believe that the binding of the protein initiates the transcription of genes necessary for bacteroid formation and nitrogen fixation. It is possible that another transcriptional regulatory protein is activated by this binding as suggested by Ronson (38).

## INTRODUCTION

There is considerable evidence that the tricarboxylic acid (TCA) intermediates, especially succinate, play a crucial role in nitrogen fixation by organisms in the genus Rhizobium. The TCA cycle has been implicated as a central pathway for carbon metabolism in bacteroids (43) and utilization of TCA intermediates has been shown to be related to symbiotic effectiveness in a variety of species (1,19,5,23,39,40). It has also been shown that succinate added to an exponentially growing culture of Rhizobium trifolii induces swelling and the pleomorphic shapes characteristic of nodule bacteroids (46,44), and that the ability to incorporate  $^{15}\text{N}$  and reduce acetylene follows in vitro bacteroid formation (45).

Further studies which will be discussed show that fumarate and malate are also able to induce bacteroid formation but seem to be less efficient. Through subsequent work, we discovered a number of other compounds which were able to induce bacteroid formation. We also did a number of binding studies which revealed that succinate binds to the surface of R. trifolii 0403 and can be competitively inhibited by the other active compounds. Upon examination of the

molecular structure of active molecules, we realized that the actual key to bacteroid formation seemed to be a receptor-effector event with the effector succinate or compounds structurally related to succinate and the receptor a part of the rhizobial cell.

Mutants defective in enzymes of the TCA cycle, such as succinate dehydrogenase and alpha-ketoglutarate dehydrogenase, form ineffective nodules (16,22). Interestingly, mutants deficient in carbohydrate metabolism generally form effective nodules (43,40). The TCA cycle intermediates succinate, fumarate, and malate are considered the most likely carbon and energy substrates used by bacteroids, since mutants defective in dicarboxylate transport (dct) grow normally on other carbon sources but are impaired in nitrogen fixation (2,8). These compounds have also been shown to support nitrogen fixation by purified bacteroids (46,5).

It has long been recognized that microorganisms possess functionally specialized permeation mechanisms which may possess a high degree of stereospecificity for the substrate being transported (13). The inducible formation of transport systems affecting the uptake of the dicarboxylic acids was originally demonstrated in *Pseudomonads* (27,11,4) and in *Aerobacter* (14,15),



however the specificity of the systems was not resolved. Kay and Kornberg in 1969 reported an inducible system specifically involved in the uptake of C<sub>4</sub>-dicarboxylic acids in E. coli (26). Mutants which lacked the system could still metabolize internal C<sub>4</sub>-acids, but failed to grow on these substances as sole carbon sources. The uptake system was shown to be specified by a gene (dct) located on the E. coli genome close to the xly marker.

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organism's ability to form effective nodules and ultimately to fix nitrogen (19). A great deal of evidence seems to indicate that C<sub>4</sub>-dicarboxylic acids produced by the plant are utilized by bacteroids in N<sub>2</sub>-fixing root nodules (5,23,39,40,). Finan et. al. (19) examined the transport of succinate in an effective streptomycin-resistant strain of Rhizobium leguminosarum. They noted high levels of succinate transport occurred when cells were grown on succinate, fumarate, or malate, and that low transport occurred when cells were grown on glucose, sucrose, arabinose, or pyruvate as the sole carbon source. A succinate dehydrogenase mutant was isolated. It was found to transport in and accumulate 400 times the external concentration of succinate. It was also found that succinate transport was completely abolished in the presence of metabolic uncouplers and that succinate transport was competitively inhibited by fumarate and malate. The conclusion of this study was that R. leguminosarum possesses a C<sub>4</sub>-dicarboxylic acid transport system which is inducible and mediates the active transport of succinate, fumarate, and malate into the cell. A cluster of four dct genes has been identified in R. leguminosarum (37). Three of these

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It was our premise in conducting this study, that there is a unique protein which binds succinate on the surface of R. trifolii 0403 and subsequently that event triggers a cascade of transcriptional events which leads to bacteroid formation and nitrogen fixation. It would seem logical that the Dct system may play a significant role in this process since it is an effective and widely conserved system in bacteria and is present and active in the rhizobia. It also seems quite likely since the Dct system is coded for on the exo megaplasmid of R. meliloti and may play a role in some unique regulatory mechanism for that plasmid. All of these factors in addition to the fact that the Dct system acts in response to environmental levels of C<sub>4</sub>-dicarboxylic acids indicate that the Dct system may play a critical role in how R. trifolii 0403 is capable of forming bacteroids in vitro and fix nitrogen in response to specific levels of succinate in the media.

## MATERIALS AND METHODS

Organism. Rhizobium trifolii 0403 (44) was used in this study.

Media. Bacteria were cultured in the chemically defined BIII medium (6) with 0.2% (wt/vol) D-mannitol (44) as the carbon source and nitrilotriacetate omitted. Sodium succinate (16.6 mM) was used as the supplement for nutrient enrichment as previously described (44). Citrate, cis-aconitate, isocitrate, alpha-keto-glutarate, fumarate, malate, oxaloacetate, acetyl salicylic acid, methyl-salicylate, 4-hydroxy-isophthalic acid, and phthalic acid were also added in 16.6 mM concentrations where described.

In vitro N<sub>2</sub> fixation. In vitro nitrogen fixation was determined as previously described (43).

Protein purification. Peripheral proteins were isolated from mid-log phase cultures of R. trifolii 0403. Twenty mls of log phase cells were pelleted and washed with phosphate buffered saline (PBS) (pH 7.0). The cells were resuspended in 0.5mls of PBS with 0.2% octyl-B-D-glucopyranoside (NOG) (Sigma Chemical Co., St. Louis, MO) added. The cells were then incubated at room temperature with periodic shaking for 15 min. The

cells were then pelleted by centrifugation and the supernatant was collected and dialyzed against water overnight at 4°C with several changes of water. The samples were then lyophilized and kept at -20°C until use.

<sup>14</sup>C-succinate binding. 1.66 uM (final concentration) of <sup>14</sup>C labelled succinate (s.p. act 59.6 mCi/mmol) were added to 5mls of a mid-log phase culture of R. trifolii 0403 which had been placed in an ice bath for 15 minutes. The cultures were kept in ice for 10 minutes and were shaken at periodic intervals. The cells were then trapped on a glass fiber filter and washed 5 times with deionized water. The filter was removed, dried and placed in a scintillation vial. Beckman liquid scintillation cocktail was added and the samples were counted on a Beckman LS 1800 scintillation counter.

Epoxy-activated sepharose 6B column chromatography. 2g of gel was used to make a final volume of 6 ml of swollen gel. Gel was swollen and washed on a filter. 100ml of 45% (wt/vol) succinate in deionized water was added and the system was titrated to pH=12.00 with 1N NaOH. This mixture was shaken

overnight at room temperature. The gel was then washed and 10 mls ethanolamine were added. This mixture was shaken overnight. Ethanolamine functioned in blocking unreacted groups on the gel. The gel was then washed and poured into a column. Purified peripheral proteins were gravity filtered through the gel and the column washed thoroughly with deionized water. Bound protein was eluted with 45% (wt/vol) succinate in deionized water.

SDS-PAGE. Mini 12.5% polyacrylamide gels (5.5 X 10 cm) were run with 3% stacking gels. Gels were stained by a highly sensitive urea-silver stain (12).

HPLC. The HPLC system was composed of a Varian Associates (Walnut Creek, CA) model 5060 microprocessor-controlled pump, a Waters Associates (Milford, MA) model 710A autosampler, a Tracor model 970 variable wavelength detector (set at 210 nm), a SynChrom, Inc. (Linden, IN) SynChropak RP-P (C-18) 6.5 particle column (250 X 4.1 mm i.d.), and a Hewlett-Packard 3388 printer-plotter automation system.

Injectons of 50 ul were made and the peak was eluted at 45 C, using the gradient defined in Table 5,

at 1.0 ml/min. The solvents were (A) acetonitrile+ 0.1% TFA and (B) water + 0.1% TFA.



## Results

### Initial studies with TCA cycle intermediates.

Table 1 shows a listing of the TCA intermediates and indicates whether or not the addition of 16.6mM amounts of the respective compounds was capable of inducing bacteroid formation and subsequent nitrogen fixation. It was found that citrate, isocitrate and cis-aconitate were not able to induce bacteroid formation, nor were cells treated with these compounds able to initiate nitrogen fixation. We did find that alpha-keto-glutarate, succinate, fumarate, malate and oxaloacetate were capable of inducing bacteroid formation and subsequently nitrogen fixation.

Further studies revealed some unusual compounds which were capable of inducing bacteroid formation and, in one case, the subsequent fixation of nitrogen. Table 2 illustrates that acetylsalicylic acid, methyl-salicylate, 4-hydroxy-isophthalic acid and phthalic acid induce bacteroid formation, and acetylsalicylic acid induces nitrogen fixation in vitro. Fig. 1 shows the molecular structure of each of the aforementioned compounds presented in Table 2. Upon examination of three dimensional molecular models

TABLE 2

Ability of structurally similar compounds to induce bacteroid formation and/or nitrogen fixation

COMPOUND	BACTEROID FORMATION	N <sub>2</sub> FIXATION
1. SUCCINATE	(+)*	(+)*
2. ACETYL SALICYLIC ACID	(+)	(-)
3. METHYL-SALICYLATE	(+)	(-)
4. 4-HYDROXY-ISOPHTHALIC ACID	(+)	(-)
5. PHTHALIC ACID	(+)	(-)

# all compounds were assayed at a concentration of 16.6 mM

+ = response was induced

- = response was not induced

Fig. 1 The molecular structures of 5 compounds which were able to induce bacteroid formation are shown here. The shaded portion of each compound represents the region of that compound which resembles succinate.

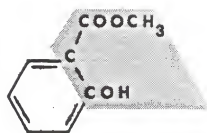
Fig.1



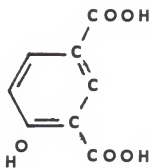
Succinic Acid



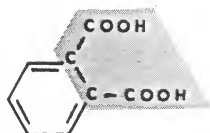
Acetylsalicylic Acid



Methyl Salicylate



4-Hydroxyisophthalic Acid



Phthalic Acid

of these compounds, we recognized that all had portions which resembled the structure of succinate as can be conjectured from the shaded portion of the molecular structure. At this point we began to suspect that succinate initially acts at the surface of rhizobia and that the interaction triggers events which lead to bacteroid formation and ultimately nitrogen fixation. We did binding studies with labelled succinate to see if we could show that succinate does bind to the surface of the cell.

Surface binding of  $^{14}\text{C}$ -succinate. To determine the nature of succinate binding accompanying the morphological and physiological transformations described above, exponentially growing cells were exposed to  $^{14}\text{C}$ -succinate under a variety of conditions and  $^{14}\text{C}$ -counts bound to cells were quantitated.

When  $1.66 \mu\text{M}$   $^{14}\text{C}$ -succinate (s.p. act 59.6 mCi/mmol) was added to untreated log phase cells, approximately 162,000 cpm were bound to the cell surfaces. We assigned that number a unity value of 1.0 (see Table 3). When cells were treated with 3.8% formaldehyde prior to the addition of labeled succinate in order to determine how the crosslinking of surface proteins would effect binding, we found that 2% of the

TABLE 3

Results of  $^{14}\text{C}$ -succinate binding studies

TREATMENT	UNITY VALUE*
UNTREATED CELLS (CONTROL)	1.0
FORMALDEHYDE TREATED	0.02
TRICHLOROACETIC ACID TREATED	0.11
AZIDE TREATED	0.07
UNLABELED 16.6 mM SUCCINATE ADDED TO CELLS TREATED WITH AZIDE AND KEPT ON ICE	0.02
16.6 mM SUCCINATE ADDED 16 HRS PREVIOUSLY	2.50
16.6 mM SUCCINATE + CHLORAMPHENICOL ADDED 16 HRS PREVIOUSLY	0.26

\* The unity value of 1.0 corresponds to 162,191 c.p.m.

label bound to the cell surfaces. We then examined the binding of the label to cells whose surfaces had been partially disrupted by 3.5% TCA. We found that 11% of the label would still bind to these cell surfaces. In an attempt to see whether active transport was playing a role, we examined binding of the label to cells which had been exposed to 100uM sodium azide. Seven percent of the label bound to the cell surfaces.

We next attempted to competitively block the binding of labeled succinate with unlabeled succinate. 16.6mM unlabeled succinate was added to cells which had been placed in 100uM sodium azide and were kept in an ice bath. The cells were washed twice with PBS and resuspended in 2 mls of PBS. 1.66 uM (final concentration) of  $^{14}\text{C}$ -succinate was then added to the cells. It was found that these cells bound up only 2% of what the control cells bound. Hence, it appeared that the cold succinate was blocking the ability of the labeled succinate to bind to the surface of the cell.

When 16.6mM unlabeled succinate was added to exponentially growing cells 16hrs prior to an attempt to bind labeled succinate to the cells, a decided increase in the ability to bind labeled succinate

occurred. In fact, 250% of the usual amount of label was bound to these cells. It is possible that more binding protein was being synthesized or more likely, that permeases had been induced.

Our binding studies suggested that a surface protein was responsible for the binding of succinate to the surface of Rhizobium trifolii 0403. In order to determine whether a surface protein was playing a role in this increase, we added unlabeled succinate 1 hr prior to label addition as before except that 50 ug/ml chloramphenicol was added along with the unlabeled succinate. It was found that the addition of chloramphenicol significantly lowered the binding response seen previously. Twenty-six percent of the unity value of label was capable of being bound with chloramphenicol blocking protein synthesis. This means that synthesis of surface proteins was inhibited as well as the synthesis of permeases.

Extraction of membrane proteins. Surface proteins were extracted from Rhizobium trifolii 0403 through the use of the nonionic detergent octyl-B-D-glucopyranoside (NOG). This detergent has been used to solubilize peripheral cell proteins from a variety of intact cells and isolated cell membranes



(30). We found that when R. trifolii 0403 cells are treated with 0.2% NOG, they lose their capacity to bind succinate and to form bacteroids in response to succinate exposure, yet the cells remain viable. We also showed that the proteins responsible for binding succinate were peripheral proteins through the experiment whose results are shown in Table 4. In this experiment,  $1 \times 10^6$  cpm of labeled succinate were added to R.trifolii0403 which had been kept in an ice bath. The cells were gently shaken for 30 min. to let the labeled succinate bind to peripheral protein receptors. The cells were then spun down and washed two times with phosphate buffered saline (PBS). The supernatants were collected and counted as the unbound fraction of labeled succinate. The cells were then treated with NOG and kept in the shaking ice bath for 15min. The cells were then spun down and washed two times with PBS. The supernatants were collected and counted as the fraction of label which was bound to peripheral proteins. The remaining cells were also resuspended in PBS and counted as the fraction of counts which had been internalized or were otherwise unextractable via NOG. The results in table 4 show that 168,780 cpm were bound by peripheral proteins and only 6,431 cpm were

Table 4

Binding of  $^{14}\text{C}$ -succinate to peripheral proteins of R. trifolii 0403

	cpm
Unbound succinate	900,660
Peripheral protein fraction (NOG extract)	168,780
Cells after NOG treatment (internalized/non- specific counts)_	6,431
Background	16

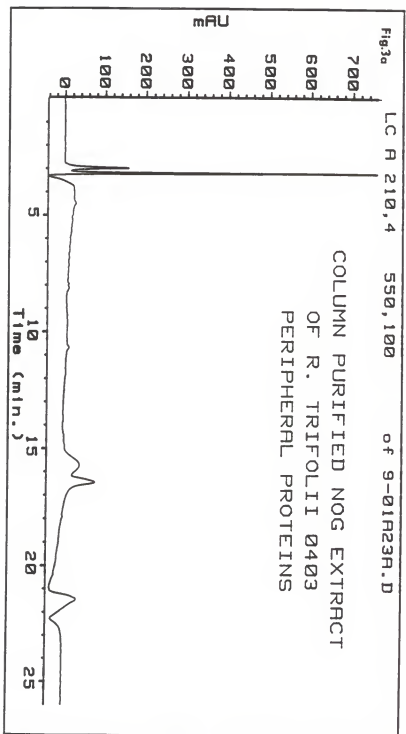
internalized or otherwise unextractable via NOG. A significant proportion of labeled succinate was bound by the peripheral proteins of *R. trifolii* 0403. Thus we knew that the protein(s) responsible for the response due to succinate exposure, were present in the NOG extract. Fig. 2a shows the protein profile of the NOG extract on 12.5% SDS-PAGE gel.

In order to remove only the protein(s) responsible for binding succinate at the surface of the cells, we bound succinate to an epoxy-activated sepharose 6B column and ran the NOG extract through. Fig. 2b shows the protein profile of the column eluent. Two bands appeared at around 55 and 65 kD respectively. In order to determine whether there were actually two succinate binding proteins or one protein was denaturing into two subunits, we prepared the column eluent for HPLC analysis. Fig. 3a shows the HPLC profile of the column eluent. Fig. 3b shows a 3-dimensional analysis of the profile. Table 5 shows the program for elution of the binding protein. The succinate binding protein peak occurs at 3.2 min. indicating that the protein is very hydrophilic. Spectral analysis of that peak revealed that it was quite pure and absorbed predominantly in the 210 nm range. This information indicated to us that the

Fig. 2. Protein profiles of total NOG extract (lane A) and HPLC purified protein (lane B) on 12.5 % mini SDS-PAGE gels stained by a highly sensitive urea-silver stain method. The arrow represents the 2 bands present in lane B. It is believed that these bands represent two subunits of one protein.

Fig. 3a. HPLC profile of the eluent from the epoxy activated sepharose 6B column. The large peak at 3.2 min. represents the succinate binding protein. The smaller peaks at 15.05, 15.15, and 21.5 min represent material which was contaminating the column eluent in very small amounts.

Fig. 3b. 3-dimensional profile of the column eluent including only the range from 2.0 min. to 3.8 min. The large peak represents the succinate binding protein. Spectral analysis of this protein showed that it was quite pure and absorbed predominantly in the 210 nm range.



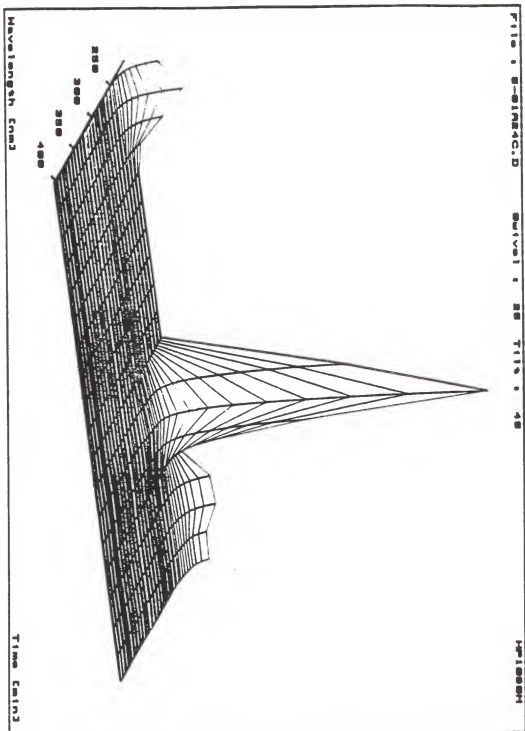


TABLE 5

Linear Gradient Program for Elution of the Succinate Binding Protein by High-Performance Liquid Chromatography

Solvent	Time (min.)					
	0	5	10	17	18	19
% A <sup>a</sup>	25	35	50	75	85	25
% B <sup>b</sup>	75	65	50	25	15	75

<sup>a</sup>Solvent A = Acetonitrile containing 0.1% trifluoroacetic acid.

<sup>b</sup>Solvent B = Water containing 0.1% trifluoroacetic acid.



peak at 3.2 min. represented one unique protein. Due to the fact that it absorbed predominantly in the 210 nm range, we know that it is composed of very few of the aromatic amino acids.

Three small peaks occur at 15.05 min., 15.15 min. and 21.5 min. These peaks represent material which was not washed away thoroughly from the sepharose column. Hence, while the column provided an excellent method for purifying the NOG extract to near purity, the HPLC indicated that for absolute purification, an additional step is required. We eluted the peak at 3.2 min. 5 times, dialyzed the material, lyophilized it, resuspended it in 50 ul deionized water and ran a 12.5% SDS gel on the HPLC purified material. We then used a particularly sensitive method for silver staining (12) due to the fact that there were minute quantities of protein present. We found that the same two bands at 55 and 65 kD were present in this gel as were present prior to HPLC. Our belief is that we purified one protein which denatured into two subunits during SDS-PAGE. We believe this since the HPLC analysis seemed to indicate that the peak at 3.2 min. was fairly pure. While it is possible that there were two very similar proteins making up this peak, the odds are that

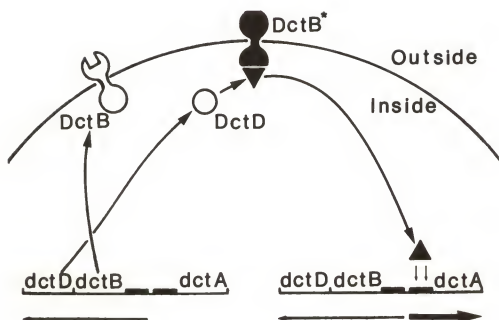
it contained only one. It would have been advantageous to run a non-reducing gel in order to determine the true nature of the peak.

## Discussion

We believe that the protein we have isolated is the protein responsible for binding succinate at the surface of the cell. It may be that this event then triggers a cascade of events which leads to bacteroid formation and ultimately nitrogen fixation. Our data fits Ronson's model (38) for the interaction of the  $C_4$ dicarboxylate gene products very well. Fig. 4 shows this model. We believe that the protein we have isolated is what is represented by the Dct B protein in the Ronson model. Our data indicates that succinate as well as other compounds which structurally resemble succinate are capable of binding to this protein. Most of the compounds which resemble succinate are capable of causing bacteroid formation which is a key event in the in vitro fixation of nitrogen. It is important to note that analogs which varied in positions of certain groups were not able to induce bacteroid formation. It is our belief, therefore, that we have found the protein which is responsible for the binding of these structurally related compounds and that it is the protein shown in Ronson's model. Our data is consistent with the data that Ronson presents in the model (38).

Fig. 4. Ronson's model (38) for the dct system. This model shows the DCT B protein at the surface of the bacterial cell. Succinate binds to this protein causingg the DCT D protein to be activated. Once the DCT D protein is active, it initiates the transcription of DCT A. Our belief is that a number of other processes are also activated leading to bacteroid formation and nitrogen fixation.

■ Succinate



REDRAWN FROM: RONSON, C.W., ASTWOOD, P.M., NIXON, B.T., AND AUSUBEL, F.M. (1987). NUCLEIC ACIDS RES 15:7921-7934.

The cascade of events that is shown in the model would be consistent with the events which lead to bacteroid formation and nitrogen fixation. The model indicates that C<sub>4</sub> dicarboxylic acids bind to this protein and trigger the cascade of events. Our binding data also suggests that transcription of the Dct A protein, a permease which transports dicarboxylic acids, may occur since there was a significant increase in succinate bound to the surface of the cells after prolonged exposure to succinate when protein synthesis was allowed to occur during this exposure. Perhaps there is another explanation for this noted increase, however, our data is none the less consistent with Ronson's model with regard to this event.

Our data show that a number of molecules which structurally resemble succinate are capable of inducing bacteroid formation, and in some cases, nitrogen fixation was initiated. Our binding data using <sup>14</sup>C-succinate shows that succinate does bind to the surface of Rhizobium trifolii 0403 in a variety of conditions. All of this data seems to indicate that there was some surface molecule which may be responsible for binding succinate (or any compound structurally resembling succinate). This receptor molecule in turn

may play a role in initiating a cascade of events which leads to bacteroid formation and nitrogen fixation. We believe that our data fit Ronson's model (38) quite well and, we believed that we were seeing evidence of the functioning of the Dct B protein in our data.

We show here a method for the purification of the peripheral proteins of Rhizobium trifolii 0403. We employed a non-ionic detergent which presumably removed the peripheral proteins of the bacterial cells. Furthermore, we show a method of isolation and purification of the protein responsible for the binding of succinate on the surface of Rhizobium trifolii 0403. We believe this protein may be a key component in the initiation of nitrogen fixation in this organism. Through the use of the method which we have devised, large amounts of the purified binding protein may be obtained. Now that the protein which appears to be responsible for bacteroid formation and for triggering events which lead to nitrogen fixation has been purified, many avenues of study have been opened for further investigation. Further study of this protein should reveal its structure and composition, should allow identification of its genes, and eventually the way in which it is capable of initiating the events

which lead to nitrogen fixation. This system seems to be an ideal model for two component regulatory systems and should prove to significant in the study of such models.

Further plans for the continuation of this study include sequencing this protein. Its sequence could then be compared to other proteins which have similar functions. Perhaps this would lead to greater understanding of how it functions to induce the cascade of events predicted by Ronson (38). Our lab would also like to prepare a monospecific antibody to the succinate binding protein to be used in a variety of assays including screening clones of transposon mutagenized organisms to determine the location of the gene for this protein.



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Identification and Characterization of a Succinate  
Receptor Protein on Rhizobium trifolii 0403.

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## ABSTRACT

Our laboratory has shown that succinate is capable of inducing rhizobia to form bacteroids in vitro, but the actual mechanism of induction has remained elusive. We now find that succinate is initially bound by a surface protein, possibly the DCT B protein which is thought to be responsible for the induction of C<sub>4</sub>-dicarboxylate transport. We have characterized the binding of [<sup>14</sup>C]-succinate to Rhizobium trifolii 0403 under normal growth conditions and after bacteroid formation. Our studies indicate succinate initially binds specifically to the cell surface, and that the ability to bind succinate is retained as cells become bacteroids. Purification of surface membrane proteins does not free bound succinate, and purified proteins retain the capacity to bind succinate. Using protein purification, SDS-PAGE analysis and HPLC, we have identified and purified the peripheral protein which is responsible for the initial binding of succinate to its surface from R. trifolii 0403. We believe that the binding of this protein is the initial event which triggers the cascade of processes which eventually leads to bacteroid formation and nitrogen fixation.