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Letter to the Editor

Possible Evolutionary Relationships of the Nitrogenase Proteins

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Summary. Comparisons of the amino acid compositions of the nitrogenase proteins from different organisms and their correlation with cross-reactivities and taxonomical data suggest an evolution within bacterial genomes rather than within plasmids. Comparisons of the amino acid compositions of nitrogenases and other ATP-ases show similarities which might be due to the evolution of these ATP-ases from a common ancestral protein.

Key words: Nitrogenases - ATP-ases - Amino Acid Compositions - SΔQ - Evolutionary Relationships

INTRODUCTION

Nitrogenases (N_2-ases) are binary enzyme complexes that catalyze the reduction of a number of compounds with a triple bond coupled to the hydrolysis of ATP, the most important reaction in vivo being

$$(1) \qquad N_2 + 6\ e + 8\ H^+ + 6\text{-}15\ ATP \rightarrow 2\ NH_4^+ + 6\text{-}15\ ADP + 6\text{-}15\ P_i$$

Other compounds which can serve as substrates are N_3^-, CN^- and C_2H_2. In the N_2-ase complex the component I contains the site of reduction, whereas the component II catalyzes the hydrolysis of ATP (for reviews see Dalton & Mortenson, 1972; Dilworth, 1974). Speculations about the evolution of these proteins are confronted with several problems:

Abbreviations: N_2-ase, nitrogenase; AAC, amino acid composition.

(a) N_2-ases are found in prokaryotes only. They are far more frequent among the more primitive anaerobic than among aerobic bacteria, and they are generally associated with apparently old proteins (e.g. ferredoxins). Thus they might be of ancient origin. However during the early evolution of those proteins the earth was supposedly covered with a reducing atmosphere containing ammonia (Bada & Miller, 1968). The energy (ATP) consuming reduction of N_2 in the presence of ammonia would have been of no selective advantage. This has lead Silver & Postgate (1973) to suggest an original depoisoning function (e.g. reduction of CN^-) for the enzyme.

(b) N_2-ases among the anaerobes are haphazardly distributed at the species level. Even within one species N_2-ase activity often is expressed only by several strains (see e.g. Mahl et al., 1965). This and the discovery of a transfer of nitrogen fixation genes (nif genes) into a non-fixing species (Dixon & Postgate, 1972), and the production of plasmids bearing nif genes (Cannon et al., 1974) lead Postgate (1974) to postulate the hypothesis, that nif genes evolved recently within plasmids and subsequently became randomly incorporated into the genetical material of various microorganisms.

In this report we wish to discuss these points.

RESULTS AND DISCUSSION

Homology of a group of proteins usually is inferred from a comparison of the amino acid sequences (see e.g. Fitch, 1973). However sequence data are scarce and mostly restricted to small proteins. Nevertheless the sequence studies have indicated that for a protein with a certain amino acid composition (AAC) usually only one of all possible sequences is found in nature. In general sequences seem to be more similar the more similar the AAC of the proteins are. This has lead to the development of empirical formulas, which use the similarity of the AAC as a screening device for a possible homology (for a critical review see Fitch, 1973). The most frequently used formula was developed by Marchalonis & Weltman (1971): the individual differences in mole per cent content of each of 17 amino acids are squared and summed, giving a value termed $S\Delta Q$ (in the following calculations the often not reported tryptophan contents are not considered). When comparing more than 100 proteins of known sequence Marchalonis & Weltman (1971) found that most of the proteins known to be highly homologous showed $S\Delta Q$ values less than 50. Only 2% of the proteins known to be heterologous had an $S\Delta Q$

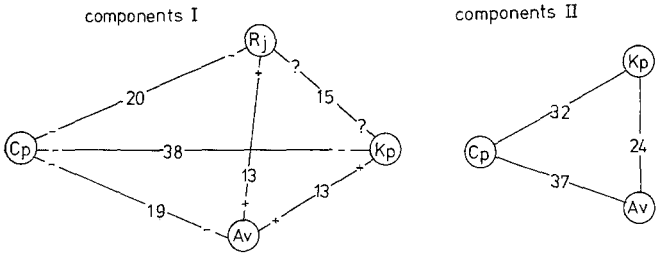

Fig.1
SΔQ values for N_2-ase components I and II (on the lines) and cross-reactivities. AACs are from Kleiner & Chen (1974) for *A.vinelandii* I and II (Av), Chen et al. (1973) for *C.pasteurianum* I and II (Cp), Eady et al. (1972) for *K.pneumoniae* (Kp), Israel et al. (1974) for *R.japonicum* I (Rj)

less than 100 and none less than 50. Keeping in mind the empirical basis of the formula as well as Fitch's critical remarks (1973), SΔQ values less than 50 suggest the possibility of an evolution from a common ancestor.

In Fig.1 the SΔQ values for the N_2-ase components I and II from various organisms are approximately represented as distances. Both proteins seem to constitute families of homologous proteins. This is not unexpected in view of their specialized function in reaction (1), and of their similar molecular properties. For both components the highest SΔQ values are obtained in the comparisons with the clostridial proteins, a situation which places the latter proteins clearly apart from the N_2-ase components of the other organisms. This is qualitatively confirmed by a comparison of the nitrogen fixation if mixtures of components I and II of different organisms are employed as catalysts (cross-reactions). In Fig.1 a positive sign close to an organism means that component I of this organism combines with component II of the organism at the end of the line to give a fully active N_2-ase. All known cross-reactions between the N_2-ase components from *Azotobacter vinelandii, Klebsiella pneumoniae* and *Rhizobium japonicum* yield a functioning N_2-ase, whereas none of their components cross-reacts with the components from *Clostridium pasteurianum* (Detroy et al., 1968; Murphy & Koch, 1971). These differences are probably due to conformational dissimilarities and thus place the clostridial N_2-ase proteins structurally apart from the others.

Taxonomical considerations point into the same direction: *C.pasteurianum* is less related to the other organisms than those are to each other (De Ley, 1968; Jones & Sneath, 1970). A number of cross-reactions has been carried out with impure N_2-ase components from various organisms (for summaries see

Dalton & Mortenson, 1972; Dilworth, 1974; Postgate, 1974). A close correlation of taxonomical relatedness with the activity of the hybrid N_2-ases has been observed. This correlation of the N_2-ase structural and functional data with taxonomy suggest an evolution of the N_2-ases along with the evolution of the bacteria rather than a random incorporation of nif plasmids into the bacterial genomes, where no such correlation would have been expected. Thus far this evidence is based only on scanty data and certainly needs further substantiation. More AACs of N_2-ases are needed as well as independent lines of evidence, e.g. sufficient immunological data.

If N_2-ases thus could be of ancient origin, relationships to other old proteins might be expected. Weltman & Dowben (1973), using SΔQ values found a close similarity in the AACs of many ATP-ases disrespective their origin. Since N_2-ases hydrolyze ATP, we compared the AACs of some bacterial and eukaryotic ATP-ases, mitochondrial proteins, some proteins from *A.vinelandii* and *C.pasteurianum* and some molybdenum proteins with the N_2-ases (Table 1). Proteins are grouped together which both have the same function and yield values of SΔQ<50 when compared to each other. Mean deviations are indicated. N_2-ases components I, N_2-ase components II, glutamine synthetases, bacterial ATP-ases and eukaryotic ATP-ases appear as rather homogeneous groups. Interestingly the table shows low SΔQ values for the cross calculations of all these groups suggesting a possible evolution from a common ancestral protein. From all the other proteins only a mitochondrial "structural protein" (which in fact might be an ATP-ase), an *Azotobacter* transhydrogenase and perhaps *Azotobacter* ferredoxin I show SΔQ values low enough to point towards a possible homology. In a recent review Dilworth (1974) using SΔQ values did not find any evidence for a relationship of the N_2-ase proteins with other bacterial iron-sulfur proteins.

It does not seem unlikely that proteins with ATP-ase activity are old proteins, who evolved from a common ancestor and later became adapted to various specialized functions (see Broda, 1971; Weltman & Dowben, 1973). In the course of the evolution some of them might have lost the ability to hydrolyze ATP (e.g. N_2-ase components I).

These considerations do not answer the question why the ability to fix N_2 is scattered so randomly among prokaryotes. Possibly nif genes are still rather common among microorganisms, but in most cases do not lead to expression of activity, because this depends on several other factors. It is known for a long time that rhizobia can only fix N_2

Table 1

SΔQ calculations for various proteins. AACs used are from the following proteins: N_2-ase components I and II as in Fig.1. Glutamine synthetases: *Escherichia coli* (Woolfolk et al., 1966), *Bacillus stearothermophilus* (Hachimori et al., 1974). Membrane bound bacterial ATP-ases: *Bacillus megaterium* (Mirsky & Barlow, 1973), *Micrococcus lysodeikticus* (Andreu et al., 1973), *Streptococcus faecalis* (Schnebli et al., 1970). Eukaryotic ATP-ases: mitochondrial coupling factor F_1 and chloroplast coupling factor CF_1 (Racker, 1970), *Acanthamoeba castelliani* actin (Weihing & Korn, 1971). Cow milk xanthine oxidase (Bray & Malmström, 1964). *Micrococcus denitrificans* nitrate reductase (Forget, 1971). Mitochondrial structural protein (Woodward & Munkres, 1966). Mitochondrial oligomycin-sensivity conferring protein OSCP (Senior, 1971). *A.vinelandii* proteins: ferredoxin I (Dervartanian et al., 1969), ferredoxin III (Yoch et al., 1969), flavodoxin (Edmondson & Tollin, 1971), cytochromes c_4 and c_{551} (Campbell et al., 1973), AMP nucleosidase (Schramm & Hochstein, 1972), pyridine nucleotide transhydrogenase (Middleditch et al., 1972). *C. pasteurianum* proteins: ferredoxin (Tanaka et al., 1966), flavodoxin (Knight & Hardy, 1967), rubredoxin (Eaton & Lovenberg, 1973), hydrogenase (Nakos & Mortenson, 1971)

	N_2-ases component I (4 proteins)	N_2-ases components II (3)	Glutamine synthetases (2)	Bacterial ATP-ases (3)	Eukaryotic ATP-ases (3)	Xanthine Oxidase (1)	Nitrate Reductase (1)
N_2-ase I	20±9						
N_2-ase II	54±9	31±6					
Gln-synth.	32±11	80±26	41				
Bact.ATP-ases	42±17	40±12	50±18	23±9			
Euk.AT-ases	43±20	48±10	50±21	26±14	24±5		
Xanthine ox.	26±5	72±14	30±1	41±16	33±12	0	
Nitrate red.	62±18	46±4	66±21	44±5	58±11	74	0
Struct.prot.	25±9	47±10	33±18	29±9	24±10	14	53
OSCP	101±5	106±12	117±30	77±21	60±11	73	151
Av proteins:							
ferredox.I	80±12	60±5	72±26	69±8	64±12	78	53
ferredox.III	293±20	216±36	318±46	255±39	279±22	298	245
flavodoxin	79±17	104±23	94±37	80±17	92±9	71	84
cyt.c_4	146±22	148±8	131±18	151±12	152±29	157	78
cyt.c_{551}	103±12	105±14	93±8	93±17	85±12	82	86
AMP nucl.	190±29	156±11	155±24	120±32	134±21	177	125
Transhydr.	34±9	65±8	38±15	30±1	40±9	40	64
Cp proteins:							
ferredoxin	438±22	447±39	453±3	485±12	491±24	447	479
flavodoxin	113±8	102±13	143±29	93±4	120±17	123	103
rubredoxin	350±31	407±6	425±5	459±41	506±56	396	415
hydrogenase	94+19	179+11	101+10	136+9	130+23	101	230

in symbiosis with the host plant, and completely loose this ability in a non-symbiotic culture. Recently Tubb (1974) reported evidence for a definitive role of glutamine synthetase in the expression of N_2-ase activity by *K.pneumoniae*. Nagatani & Brill (1974) on the other hand found a requirement of molybdenum for the induction of component I synthesis in *A.vinelandii*.

The scheme for a possible evolutionary pathway for the N_2-ases as presented here can thus only be regarded as a speculative suggestion, but might help to suggest new comparisons and investigations, which then could lead to a better understanding of the problem.

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