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# REVIEW ARTICLE

# Evolutionary extremophilic Archaeal domain of life

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The discovery of varied microorganisms under extreme habitations led to the unlocking of the evolutionary secret of the biokingdom including the search for the progenitor of the most primitive form of life on Earth and in other planets. Fuelled by the limitless possible commercial applications of the extremozymes derived from their organisms, the industry anxiously awaits the results related in designing superior enzymes for commercial applications.

THE geological time scale for the evolution of the living world whose history spans almost with the formation of this Earth shows (Table 1) the existence of microorganisms in the era of Archaean. The gap of 1.1 billion years between the creation of the Earth and Archaeal period witnessed severely harsh conditions compared to the present-day ecosystem. However, the stage was almost set for the chemical evolution. It cannot be doubted that suitable portions of inorganic matter occurring amidst favourable surroundings (anoxic, intense UV radiation from the young Sun without ozone layer) may by the influence of Nature's agent of which heat and moisture are the chief, receive an arrangement of these parts that foreshadows cellular organization and thereafter pass to the simplest organic state and finally manifest the earliest movement of life. Life thriving about 3.5 billion years ago in an ambience of extreme temperature under anoxic conditions had to be surely quite different from the thermolabile life forms of today. The hyperthermophilic primitive life form does not fit into the Eucaryote–Procaryote dichotomy. In 1965 Zuckerkandl and Pauling suggested that the systematics of life forms should be based on the most fundamental molecular criteria rather than on the classical cytological phenotype criteria[1]. The confluence of advances in microbiology, enzymology and molecular biology has allowed the distinctions of different forms of life as interpreted by Carl Woese[2] which led to the proposal that living beings on this planet should be classified into three domains: (Figure 1).

— Archaea (Greek, primitive; previously archaeobacteria)
— Bacteria (Eubacteria) and
— Eucarya (Eucaryotes).

Table 1. Geological time scale (broken line is the marker for the anoxic to oxic transition)

| Era | Years (BP) | Important events | Life |
|---|---|---|---|
| Big bang (Universe) | 15-20 b | Stars | – |
| Earth | 4. 6 b | Expanding universe | – |
| Archaean | 3. 5 b | – | Evidence of micro-organisms |
| Precambrian | 1500 m | Planet cooled | Primitive algae |
| Cambrian | 570 m | – | Algae |
| Paleozoic | 286 m | $O_2$, ancient life | Modern insect |
| Mesozoic | 144 m | Continental drift | Dinosaurs reach peak/extinct flowering plants |
| | 38 m | Rise of Himalayas | – |
| Cenozoic | 0. 01 m | Warmer climate (end of ice age) | Age of human |

b, billion; m, million.

Studying the properties of Archaea might help us to trace back the nature of the last common ancestor which Woese called 'the progenote'. In the Archaean age the environmental conditions emphasized the difficulty of imagining the generation and concentration of complex organic molecules to compose the first form of life[3]. The smallest constituent of life is a cell surrounded by its environment where the free energy inside the cell should be far from equilibrium. The minimum requirements for a living cell are:

(i) Maintenance of a cytoplasmic volume separated from the bulk medium,
(ii) Its ability to perform some catalytic conversions,
(iii) Energy, and
(iv) Reproduction with the passage of information to ensure that offspring is relatively similar to the parent.

## Progenitor to protocell

Cairns-Smith and Russell have proposed a simple mineral origin of life[3,4]. It has been argued that the primitive springs of superheated water were broadly comparable to present-day blacksmokers of the hydrothermal vents, which release enormous amounts of hydrogen sulphide and metal sulphides. These metal sulphides predominantly contain iron-sulphide with effective incorporation of nickel, tungsten and molybdenum as sulphide precipitated on the deep Hadean (primordial) ocean floor. As the gelatinous sulphide bubbles aged and were inflated beyond their strength, they budded producing daughter bubbles by the precipitation of new membrane. Modification of these metal sulphide membranes might take
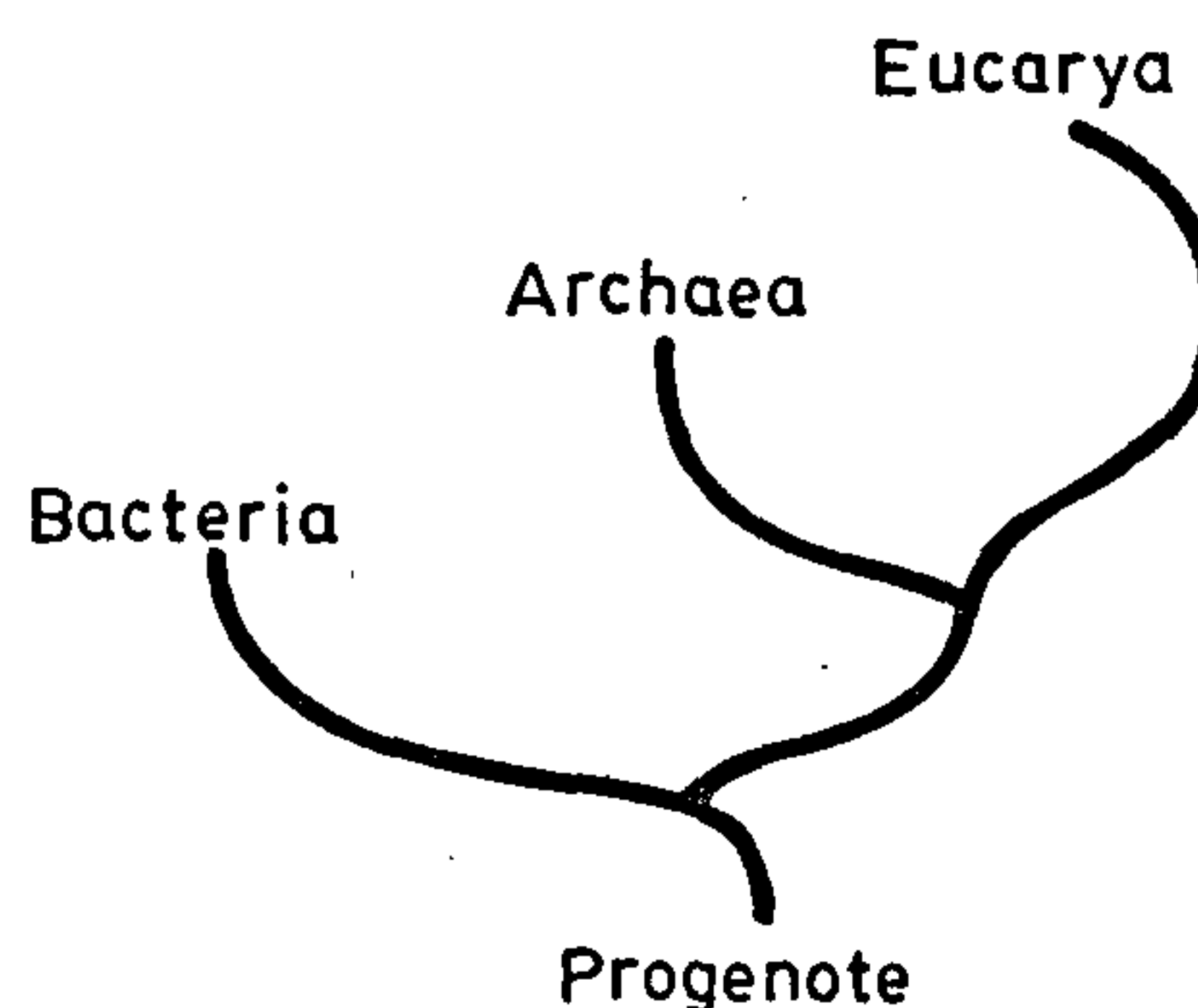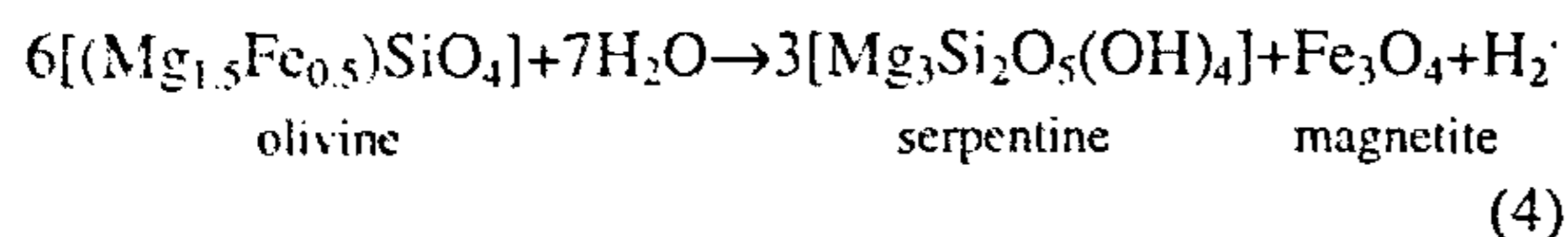


Figure 1. Woese's classification of biokingdom.

place in the presence of sulphide, polysulphide and thiolate as ligands and also as the redox partners for the synthesis of protoferredoxins. This complex sulphidic membranes may be regarded as the progenitor to proto-cells[4]. It was envisaged that an iron sulphide membrane separating a volume from the external medium could grow by hydrothermal inflations and subsequently catabolize hydrothermal abiogenic organics trapped on the inner side of the iron sulphide membrane. The modified Fe–S clusters present in the membrane cleave hydrophobic compounds to hydrophilic moieties which were translocated by the proton motive force inherent in the acidic Hadean ocean containing dissolved $CO_2$ to the sulphidic alkaline interior of the protocell. The organic take over of the sulphidic membrane would encapsulate protocells which would grow by osmosis. This protocell may have the potential to carry out catalytic conversions and perhaps may act as genetic information in the sense that the composition of the membrane could control the nature of components which can be inserted into the cell or permeate through the cell. This information may be transferred to the daughter cells. The membrane of the primitive protocell thus holds information and at the same time controls its semipermeable nature. The Fe–S systems are suggested as primordial catalysts and they can play the role of NADP $(H)$[5,6]. The versatility of their redox activity may be enhanced by the incorporation of other metals like Ni, Cu, Mo or W. Iron sulphide system in the presence of thiols and sulphides have rich redox chemistry principally linked to the following reactions,

$$Fe^{2+} \rightarrow Fe^{3+} + e^-, \tag{1}$$

$$2S^{2-} \rightarrow S_2^{2-} + 2e^-, \tag{2}$$

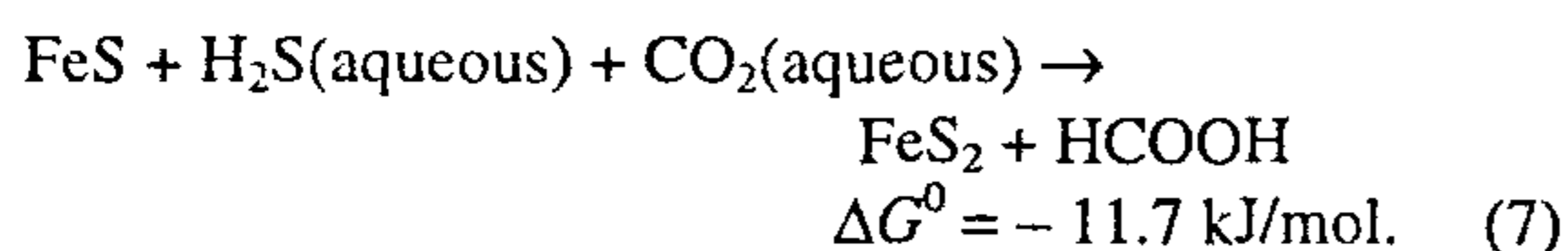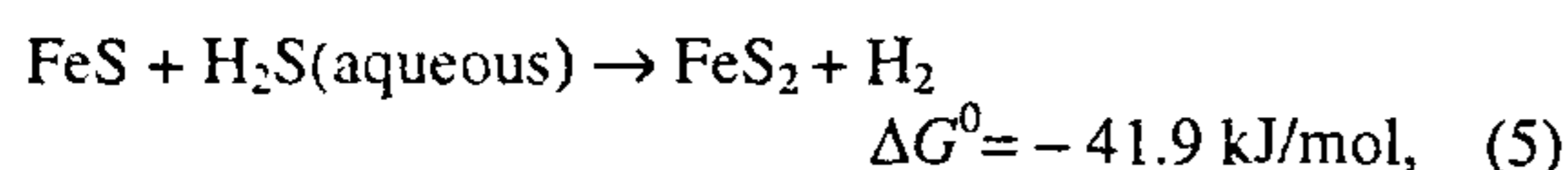$$RS^- \rightarrow 1/2\ RSSR + e^-. \tag{3}$$

These reactions can readily lead to the formation of proferredoxin types of molecules along with heterometal (Ni, Cu, Mo, W) sulphidic aggregates which had the potentiality to catalyse primitive redox based reactions under 'Fischer Tropsch' conditions. These systems can

function as hydrogenase involving acidic oceanic water activity and Fe centre within the membrane which in turn can be reduced back by the involvement of RS⁻ in the interior part of the membrane (Figure 2). Silicate-rich crust of the early Earth can also provide the necessary hydrogen as known in the case of ease of oxidation of rocks by water[7-9]:

$$6[(Mg_{1.5}Fe_{0.5})SiO_4]+7H_2O \rightarrow 3[Mg_3Si_2O_5(OH)_4]+Fe_3O_4+H_2$$

olivine  serpentine  magnetite

(4)

Wächtershäuser developed the relationship between sulphur chemistry and life[10-12]. He demonstrated the first source of energy and reducing power for the reduction of $CO_2$ in relevance to the origin of life:

$$FeS + H_2S(aqueous) \rightarrow FeS_2 + H_2$$
$$\Delta G^0 = -41.9 \text{ kJ/mol}, \quad (5)$$
$$CO_2(aqueous) + H_2 \rightarrow HCOOH(aqueous)$$
$$\Delta G^0 = +30.2 \text{ kJ/mol}, \quad (6)$$

$$FeS + H_2S(aqueous) + CO_2(aqueous) \rightarrow$$
$$FeS_2 + HCOOH$$
$$\Delta G^0 = -11.7 \text{ kJ/mol}. \quad (7)$$

The simplistic model for the proton motive force takes into account the energy transduction and its conservation across the protocell membranes. De Duve proposed that thioester compounds could be available in the primitive sulphide rich organic membranes[13] which can initiate phosphate based metabolic cycle involving equations (8)–(9):

$$R'SCOR + P_i^- \rightarrow R'SH + RCOP^{--}, \quad (8)$$

$$RCOP^- + P_i^- \rightarrow RCOOH + PP_i^{2-}. \quad (9)$$
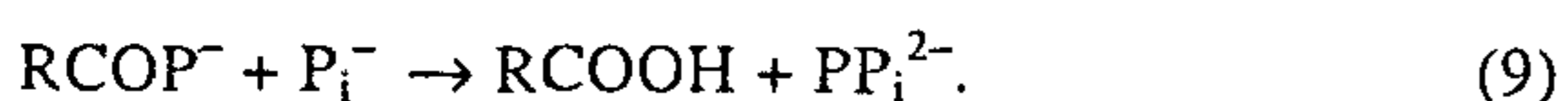
The production of acyl phosphates and pyrophosphates may be regarded as the possible precursors of ATP. Thus for the development of metabolism, the three important components like a proton gradient, electron transfer agents and thioester bonds capable of yielding polyphosphates are incorporated in this type of primitive membrane which are the minimum requirements for the then current level of evolution. The advantages of this hypothesis which fit well with the conditions prevalent on the early Earth are either well known or have been tested in the laboratory. This suggests that life emerged as a natural consequence of the drive for equilibrium between the Earth's crust and hydrosphere equilibrated with the atmosphere.

The dichotomy between the heterotrophic and autotrophic theories of the origin of life is then a matter of interpretation. Thus if the overall hydrothermal system
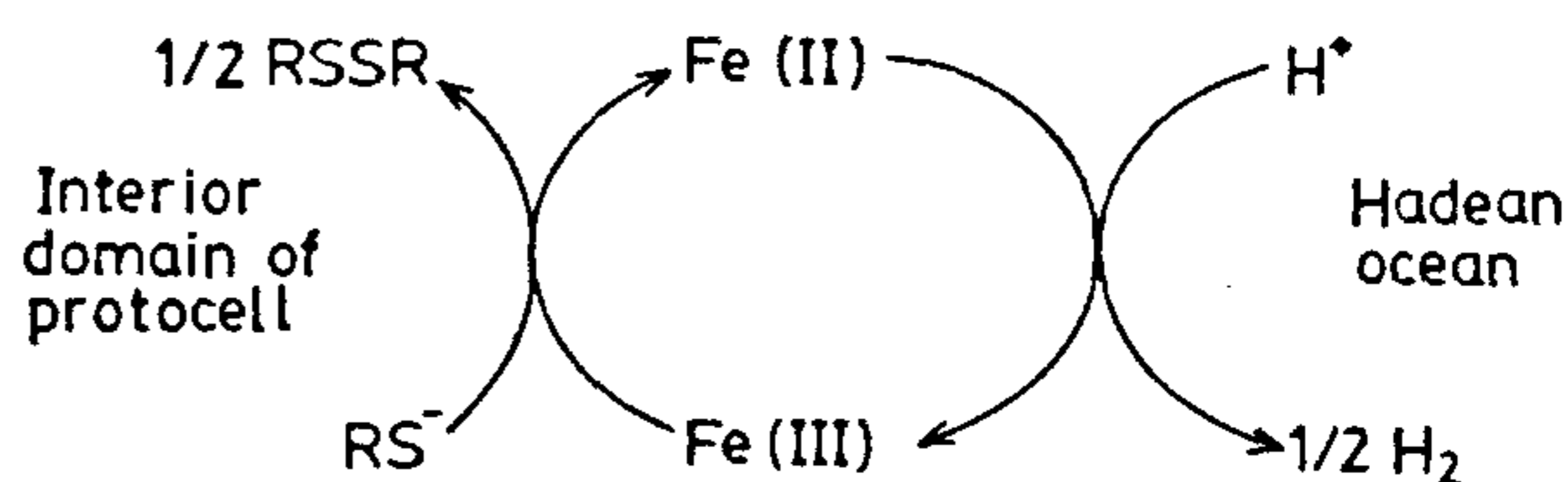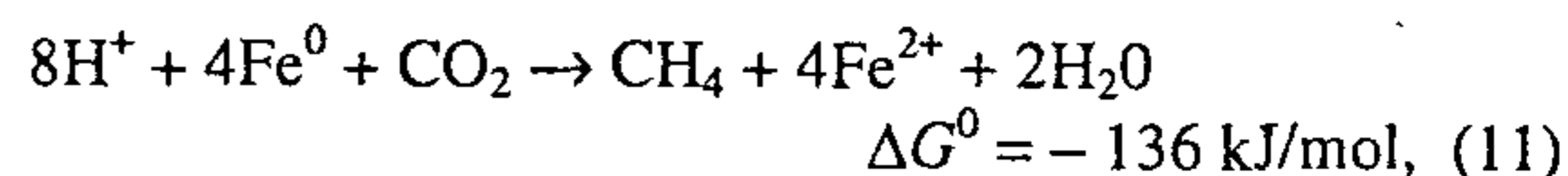


Figure 2. Natural hydrogenase reaction, the Fe–S membrane may contain traces of Ni.

with the formation of protocell membrane development is the first approach to life then it is autotrophic, otherwise the different metal sulphidic system which catalyses hydrogenation of CO to hydrocarbons by 'Fischer Tropsch' synthesis and by the hydrogenation of $N_2$ to yield ammonia by Haber-Bosch process and the generation of prebiotic formaldehyde from $CO_2$, CO and water vapour by solar photons leading to the building of more complex organic molecules may lead to the heterotrophic form of life.

Early anabolic synthesis of abiogenic organics utilized CO, $CO_2$ and/or HCHO as the most simple carbon sources. In this context the following thermodynamically feasible reactions

$$4H_2 + CO \rightarrow CH_4 + 2H_2O \quad \Delta G^0 = -139 \text{ kJ/mol}, \quad (10)$$

$$8H^+ + 4Fe^0 + CO_2 \rightarrow CH_4 + 4Fe^{2+} + 2H_2O$$
$$\Delta G^0 = -136 \text{ kJ/mol}, \quad (11)$$

are important. The gap between this mineral origin of life and the later evolutionary development of the 'RNA world'[14-18] is difficult to bridge, but all the ingredients like ribose from the condensation of formaldehyde, adenine and uracil from the condensation of HCN, essential amino acids and phosphates were available for the transformation of earliest version of the present day life[19].

## Archaea

As hyperthermophily was a relic of Archaeal environmental conditions and the anoxic state was the second important trait then in today's conditions prevalent on the Earth the survival of the most primitive form of life is possible only under similar extreme conditions. Deep sea hydrothermal vents fit well with this environmental description. Microorganisms that grow at and above 100°C were discovered a decade ago and about 20 different genera are now known[20-25]. Among these all but two genera are classified as Archaea (Figure 1). The exceptions are the bacterial genera *Thermotoga* and *Aquifex*. All have been isolated from geothermal heated environments including deep sea hydrothermal vents. These include three methanogenic genera and a unique

sulphate-reducing genus termed *Archaeoglobus*. Besides these chemolithoautotrophs, the majority of Archaea are obligate heterotrophs. They use proteinaceous growth substrates and some can also utilize carbohydrates. Most of these organisms are obligately dependent on elemental sulphur for growth which they reduce by respiratory type mechanism. Only a few species can grow without elemental sulphur by fermentative type metabolisms, although these organisms can also reduce elemental sulphur if it is added to the growth medium.

A primitive slice of life once isolated from its natural habitat and trapped and buried beneath soil for millions of years may gradually adopt newer habitation concomitant to changes in their lifestyle incorporating changes in their genetic bases. The scarcity of food material forced them to adopt exotic metabolism and very slow rates of reproduction (100 years!). It is estimated that microbial activity in the deep Basin occurs at a rate $10^{-15}$ to that in the surface soil[26]. Thus primitive forms of life under duress are capable of prolonged 'suspended animation' which may pass the Archaeal age via all the subsequent geological periods to exist even today under extreme conditions of habitation which is even extended to arctic or antarctic weather.

Depending on the habitation, Archaea are presently classified as hyperthermophiles, barophiles, halophiles, acidophiles, alkaphiles and psychrophiles. The biodiversity of such environment is illustrated in Table 2. One must keep in mind that ambient mesophilic conditions appropriate to our survival and functions may be extreme to microorganisms from unusual habitat.

## Metabolism in Archaea

### Autotrophs

Most methanogenic Archaea generate energy by the formation of methane from $CO_2$ and $H_2$ and the carbon is eventually fixed into acetyl-CoA. Therefore carbohydrate synthesis rather than catabolism is the main direction of central metabolism. Nature has evolved three major strategies for methanogenesis: reduction of $CO_2$, reduction of a preformed methyl group and its oxidation to acetate. The second stage of the strategy, i.e. the methyl reductase reaction is unique for the methanogens and has not been found elsewhere in Nature. In the first step of the $CO_2$ fixation, the enzyme, *N*-formylmethanofuran dehydrogenase (FMDH), catalyses the first step in the conversion of $CO_2$ to methane utilizing another substrate, methanofuran (MFR):

$$CO_2 + MFR^+ + H^+ + 2e \leftrightarrow CHO\text{–}MFR + H_2O. \quad (12)$$
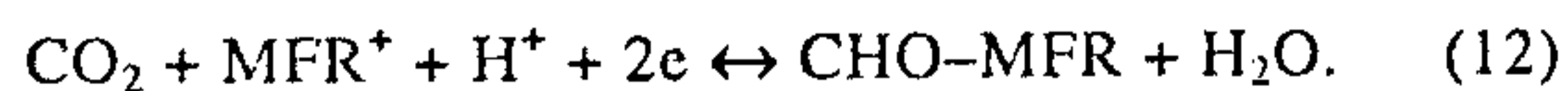
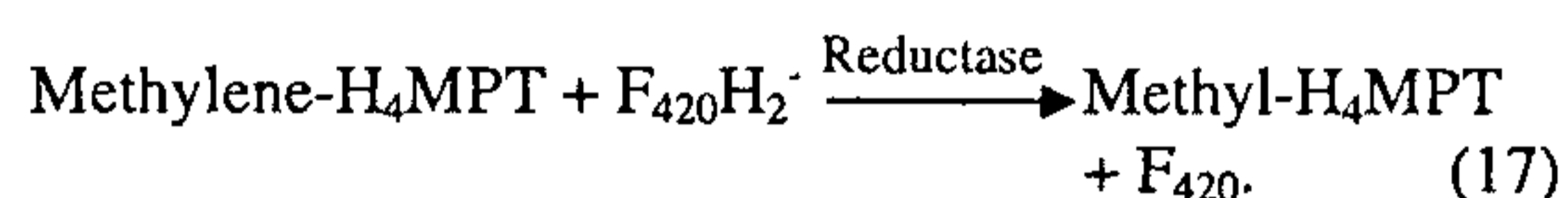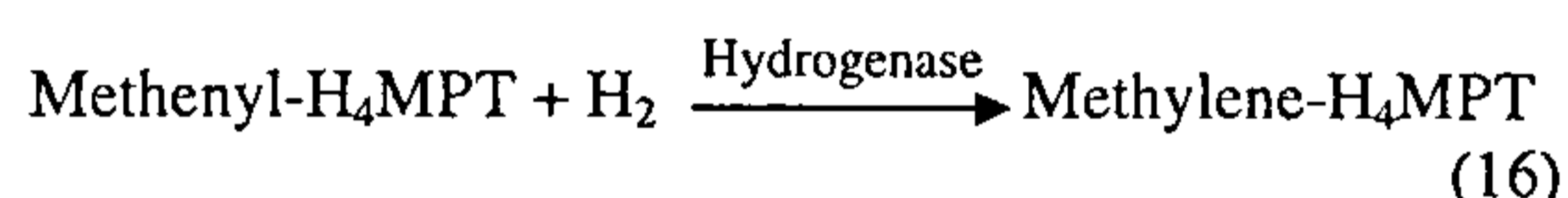The physiological electron donor for this enzymatic reaction is not known. In the next stage, the formyl group of formylmethanofuran is transferred to a pterin by formyl transferase:
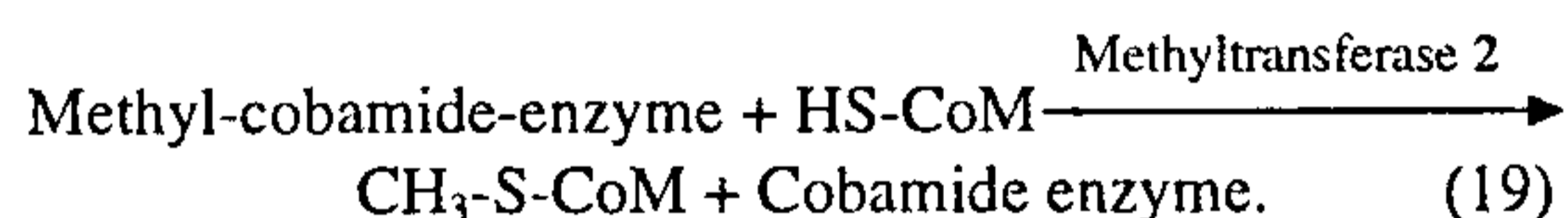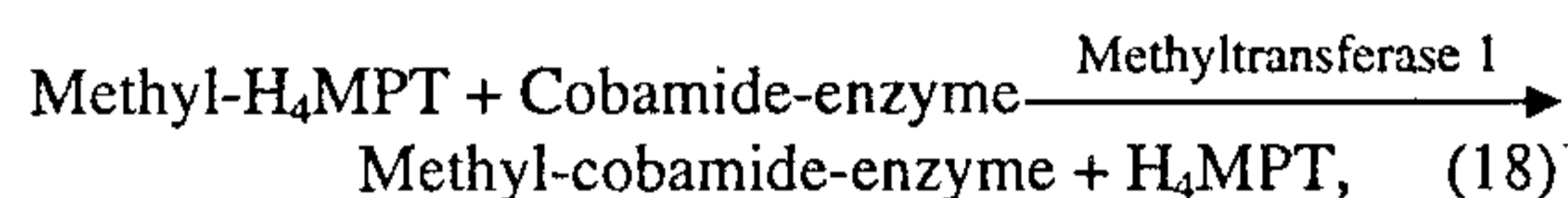
$$\text{Formyl-MFR} + H_4MPT \xrightarrow{\text{Formyl transferase}} \text{Formyl-}H_4MPT + MFR. \quad (13)$$
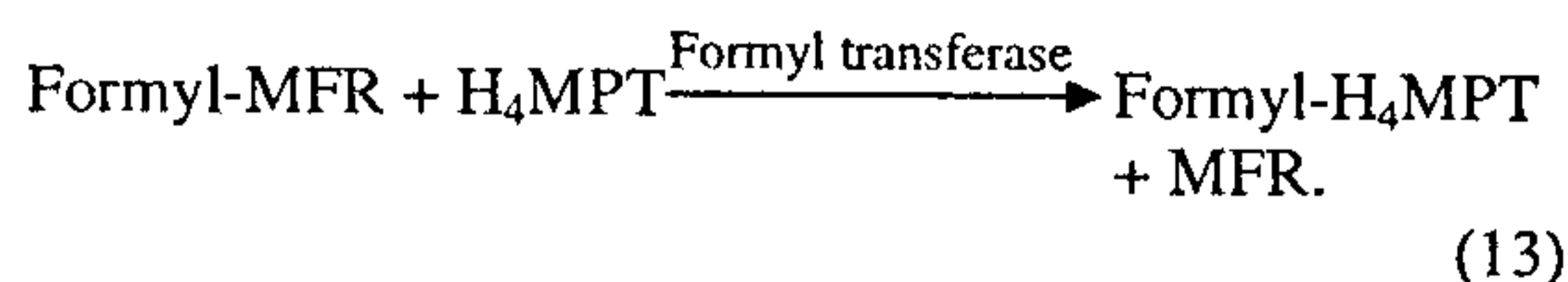
Subsequently water is removed from the formyl group by cyclohydrolase to form methenyl-$H_4MPT$:

$$\text{Formyl-}H_4MPT \xrightarrow{\text{Cyclohydrolase}} \text{Methenyl-}H_4MPT + H_2O. \quad (14)$$
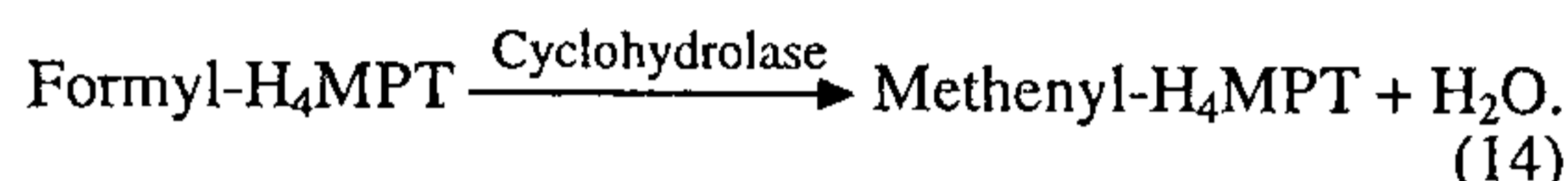
The product of this reaction responds to an oxidoreductase reaction where $F_{420}$, which is an 8-hydroxy-5-deazaflavin derivative, functions as a coenzyme for hydrogenase and also as a major electron carrier in methanogens as:

$$\text{Methenyl-}H_4MPT + F_{420}H_2 \xrightarrow{\text{Oxidoreductase}} \text{Methylene-}H_4MPT + F_{420}, \quad (15)$$

$$\text{Methenyl-}H_4MPT + H_2 \xrightarrow{\text{Hydrogenase}} \text{Methylene-}H_4MPT \quad (16)$$

$$\text{Methylene-}H_4MPT + F_{420}H_2 \xrightarrow{\text{Reductase}} \text{Methyl-}H_4MPT + F_{420}. \quad (17)$$
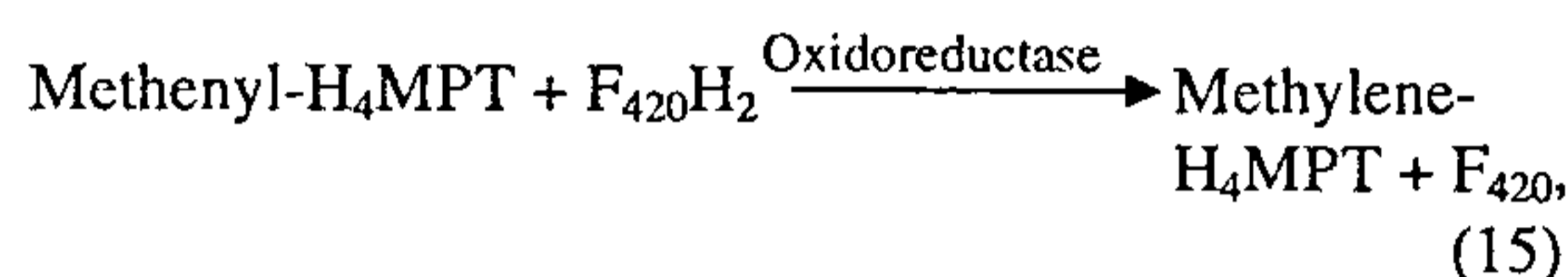
It is proposed that there are two successive methyl transferase steps between methyl-$H_4MPT$ and HS-CoM[27]:

$$\text{Methyl-}H_4MPT + \text{Cobamide-enzyme} \xrightarrow{\text{Methyltransferase 1}} \text{Methyl-cobamide-enzyme} + H_4MPT, \quad (18)$$

$$\text{Methyl-cobamide-enzyme} + \text{HS-CoM} \xrightarrow{\text{Methyltransferase 2}} CH_3\text{-S-CoM} + \text{Cobamide enzyme.} \quad (19)$$

Cobamide enzyme = a cobaltoenzyme involving $\{CH_3\text{-Co}^{III}(N)_4\}$ moiety,

HS-CoM = 2 mercaptoethanesulphonic acid,

$CH_3$-S-CoM = 2-(methyl thio) ethanesulphonic acid.

The product of this enzymatic reaction is the true substrate for the methyl reductase catalysed reaction[28]:

$$CH_3\text{–S–CoM} + \text{HS-HTP} \xrightarrow{\text{Methylreductase}} CH_4 + \text{CoM–S–S–HTP.} \quad (20)$$
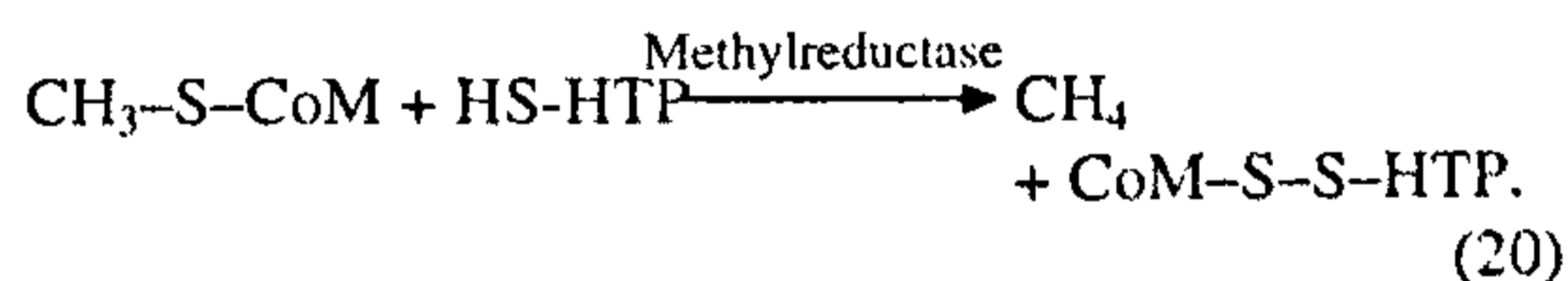
HS–HTP = 7-mercaptoheptanoylthreonine phosphate,

CoM–S–S–HTP = Heterodisulphide.

Table 2. Microorganisms under extreme environments[87]

| Extreme condition | Microorganism | Habitat | Extreme growth conditions | Metabolic characteristics |
|---|---|---|---|---|
| High temperature | Pyrococcus furiosus | Geothermal marine sediments | 100°C | Anaerobic heterotroph |
| Cold temperature | Bacillus TA41 | Antarctic sea water | 4°C | Aerobic heterotroph |
| High pressure | Methanococcus janaschii | Deep sea hydrothermal vent | 250 atm 85°C | Growth and methane production stimulated by pressure |
| High pH | Clostridium paradoxum | Sewage sludge | pH 10.1 56°C | Anaerobic heterotroph |
| Low pH | Metallosphaera sedula | Acid mine drainage | pH 2.0 75°C | Facultative chemolithotroph |
| High salt | Halobacterium halobium | Hypersaline waters | 4–5 M NaCl | Aerobic heterotroph |

The link between the methyl reductase and $CO_2$ fixation was found to be CoM–S–S–HTP. It has been observed that the addition of the heterodisulphide to reaction mixtures stimulated formylmethanofuran synthesis from $CO_2$ 40-fold[29], presumably by the heterodisulphide activation of a low potential electron carrier involved in $CO_2$ reduction although the detailed mechanism of this reaction remains obscure[30].

All autotrophic methanogens and sulphate-reducing Archaeon of the genus *Archaeoglobus* used a reductive acetyl-CoA pathway:

$$CO_2 \xrightarrow{6\,H} CH_3\text{–}H_4MPT$$
$$\searrow$$
$$\rightarrow CH_3COSCoA.$$
$$CO_2 \xrightarrow{2\,H} CO. \qquad\qquad (21)$$
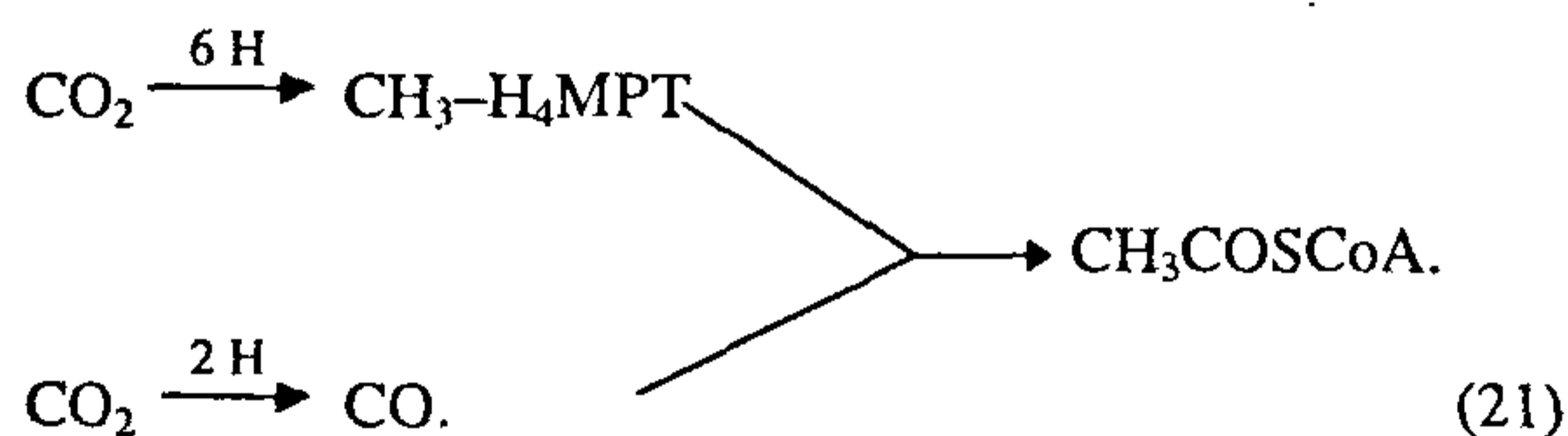
The reactions are catalysed by two important enzymes like carbon monoxide dehydrogenase (CODH) and acetyl-CoA synthase (ACS). The formation of acetyl-CoA requires two molecules of $CO_2$ which are separately reduced to form an enzyme bound carbonyl group via CODH and the other to a tetra-hydropterin bound methyl group, the synthesis of which has been briefed earlier. The condensation of the carbonyl unit and the methyl unit to acetyl-CoA are both catalysed by bifunctional CODH/ACS. The acetyl-CoA is further reductively carboxylated to pyruvate[31]. No methanogen has yet been reported to have complete oxidative or reductive citric acid cycle. The partial version of the cycle which can be observed fulfils an anabolic road for biosynthesis[32].

For the sulphur-dependent Archaeon of *Sulfolobus* genus there is enzymological and metabolic evidence that $CO_2$ is fixed via a reductive citric acid cycle[33,34].

Energy conservation in methanogen is the anaerobic respiration in which the oxidation of hydrogen is coupled to the reduction of $CO_2$. For sulphur-dependent Archaea the reduction of sulphate or elemental sulphur instead of $CO_2$ occurred. All the three inorganic electron acceptors are weak oxidants and are not exergonic enough to allow the synthesis of ATP coupled to these reactions. In addition, the hydrogen partial pressure found in most parts of anaerobic environment is only 1000th of standard conditions. The free energy changes under nonstandard conditions associated with $H_2$-dependent reductive reactions are therefore far less. Another major energetic problem is the 8 electron reduction of $CO_2$ to $CH_4$ and of sulphate to $H_2S$. Both are involved in endorgenic partial reactions like reduction of $CO_2$ to formaldehyde level and reduction of sulphate to the sulphite level at the first stage. This means that initially energy has to be spent before energy can be obtained by subsequent exorgenic partial reactions. The energy barrier is overcome by reverse electron transport in the reduction of $CO_2$ to the formyl level catalysed by membrane bound oxidoreductase and in the case of the substrate sulphate it binds with an ATP forming a sulphuric acid–phosphoric acid anhydride which then can be reduced by $H_2$. The use of elemental sulphur as terminal electron acceptor is made by the heterotrophic Archaeal organisms.

## Heterotrophs

Heterotrophic Archaeal organisms are generally obligately dependent on elemental sulphur for growth. Better studied among these organisms are of the genera *Pyrococcus* and *Thermococcus*. They grow by fermentative type metabolism but can also reduce elemental sulphur if it is added to the growth medium. Hyperthermophilic Archaea such as *Pyrococcus* and *Thermococcus* are re-

garded as the most slowly evolving of all known organisms[2]. Thus from evolutionary perspective, detailed studies of the enzymes and proteins from these two genera have been made. The primary fermentation pathways in these organisms emphasized the need to describe the physiological functions of the oxidoreductase type enzymes that have been characterized from these species. The recent developments in the structure of these enzymes and their specific roles may directly be related to the evolutionary aspect.

The pathways of electron flow from carbohydrates or peptides to hydrogen and elemental sulphur are summarized in Figure 3. Among the enzymes mentioned in Figure 3 GAPOR, FOR, VOR and IOR are unique and are not present in mesophilic anaerobes. It was initially believed that the hyperthermophilic Archaea contained a ferredoxin-dependent, Entner–Doudoroff type pathway, in which AOR catalysed the oxidation of glyceraldehyde[35]. More recently, a modified version of this pathway retaining the activity of AOR was proposed based on $^{13}C$ labelling study[36]. However using $^{13}C$ NMR spectroscopy, the predominant route for sugar fermentation in *P. furiosus* was shown with the non-involvement of any role of AOR in an unusual Embden–Meyerhof pathway[37]. However, the extremely low activity in cell-free extracts of *P. furiosus* of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) resulted in the isolation and purification of a new enzyme, glyceraldehyde-3-phosphate ferredoxin oxidoreductase (GAPOR)[38]. The production of 3-phosphoglycerate by GAPOR rather than 1,3-biphoshoglycerate by GAPDH was consistent with the growth of *P. furiosus*[39]. In addition to GAPOR, another oxidoreductase type enzyme, pyruvate ferredoxin oxidoreductase (POR) is involved for the metabolism of sugars to acetate in *P. furiosus*. A remarkable feature of the unusual Embden–Meyerhof type fermentative pathway in *P. furiosus* is that the electrons generated in the oxidation processes are funnelled to ferredoxin. The oxidation of the reduced ferredoxin is coupled ultimately to the production of $H_2$. If elemental sulphur is present then it leads to the production of $H_2S$. However the terminal hydrogen evolving hydrogenase enzyme has been shown to accept electrons not directly from reduced ferredoxin but via the physiological donor NADPH. The reduction of NADP using ferredoxin as the electron donor is catalysed by the enzyme ferredoxin: NADP oxidoreductase (FNOR)[40,41]. Interestingly, the hydrogenase enzyme has been termed as sulphydrogenase reflecting its dual catalytic activities. When elemental sulphur or its soluble version as polysulphides (PS) is absent, the job of FNOR is to catalyse the reduction of NADP only and sulphydrogenase catalyses the production of $H_2$. But in the presence of $S^0$ or PS the former (FNOR) functions as FNOR and also as sulphide
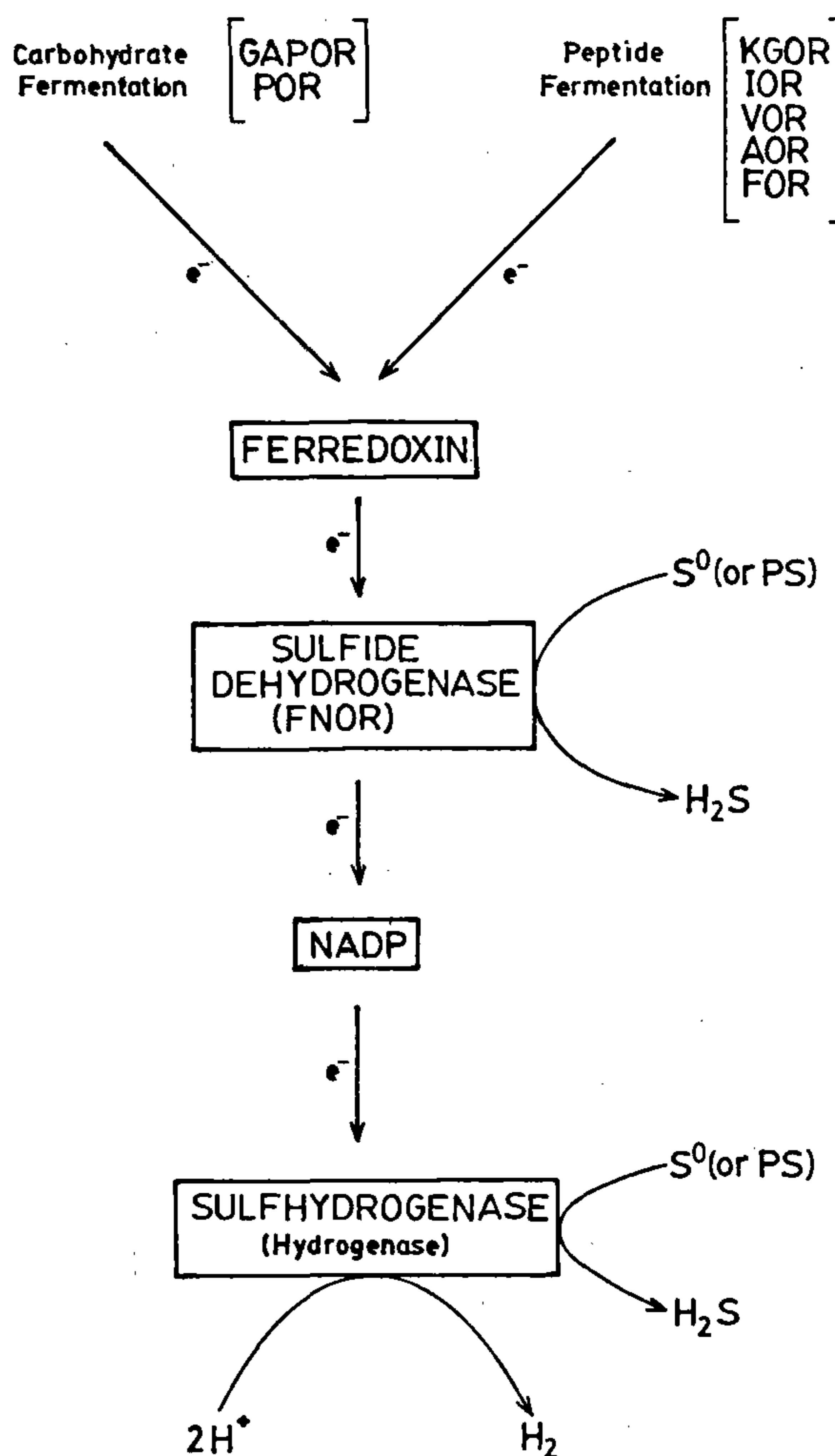


Figure 3. Proposed electron flow pathway of metabolisms by hyperthermophilic Archaea[44].

dehydrogenase and the hydrogenase displayed its dual catalytic activities (Figure 3). Interestingly, a small redox protein, rubredoxin which is present in *P. furiosus*[42], has been shown to stimulate significantly $S^0$ reducing activity but not the $H_2$ evolution activity of the hydrogenase *in vitro*[43]. However, its physiological role is not yet known[44].

Species from *Thermococcus* genus possess analogous enzymes to that from *P. furiosus*. However, these organisms also grow well in the absence of carbohydrates by the fermentation of peptides and they contain high intracellular protease activities[45–50]. *Thermococcus* strain ES-1 only metabolizes peptides[51,52] and *Thermococcus litoralis* preferentially utilizes peptides even in the presence of sugars[53].

In contrast to the mesophilic anaerobic Bacteria[54,55], these $S^0$ dependent hyperthermophiles appear to use

only transaminases for the conversion of amino acids derived from peptides[52]. The hyperthermophilic version of glutamate dehydrogenase (GDH) has been purified from several species of *Pyrococcus* and *Thermococcus*[56–62]. The GDH regenerates the amino acceptor 2-keto glutarate from the glutamate produced by transamination reaction[63,64]. The hyperthermophilic sulphur reducing organisms, in contrast to mesophiles, contain three distinct types of 2-keto acid ferredoxin oxidoreductases involved in amino acid metabolism (Figure 3). IOR[65] (indole pyruvate), VOR[66] (2-ketoisovalerate) and KGOR[67] (2-ketoglutarate) convert the transaminated forms of the amino acids to their acyl (or aryl)-CoA derivatives. In *P. furiosus* the acetyl-CoA can be used for ATP synthesis by unique enzyme ACS, which converts acetyl-CoA, ADP and phosphate into CoA, acetate and ATP in one step[68]. The corresponding aryl-CoA derivative can be used for energy conservation involving isoenzyme of ACS[69]. These isoenzymes use the products of the IOR, KGOR and VOR reactions to generate ATP and the corresponding acid. Thus different organic acids in addition to acetate have been detected in the growth media of these organisms[52,70,71].

In the amino acid fermentation the 2-keto acid oxidoreductases in addition to catalysing the oxidation of 2-keto acid also decarboxylate to yield aldehyde[72]. The aldehydes thus produced are oxidized to the corresponding acid catalysed by aldehyde oxidoreductase (AOR) which has a broad substrate specificity[66]. Another aldehyde oxidizing enzyme, formaldehyde ferredoxin oxidoreductase (FOR) has also been purified from both *T. litoralis*[53] and *P. furiosus*[73]. The FOR enzyme only oxidizes $C_1$–$C_3$ aldehyde and apparently plays a role analogous to AOR but its true function is not yet clear. In addition, a third aldehyde utilizing enzyme, alcohol dehydrogenase was found to be present in *T. litoralis*[62] and *Thermococcus ES-1* (ref. 52). ES-1 was isolated from the gut of a tube worm (*Paralvinella* sp.)[51] which lives in close vicinity to deep sea hydrothermal vents. The involvement of AOR, FOR and ADH to deal with aldehyde produced is thought to be depending on the redox status of the cell. Thus aldehyde can be disposed of to generate alcohols involving NADPH via ADH or they can be catalytically oxidized by AOR to produce acids and reduce the electron carrier, ferredoxin. Confirmation of all these fermentative pathways has yet to be made by metabolic leveling studies. It is interesting to note that the pathway for metabolism of sugars in *P. furiosus* contains other unusual enzymes such as ADP-dependent hexosephosphatekinases[37]. These unusual pyrophosphate-dependent kinases have been found in some anaerobic Bacteria and Eucarya[74]. Interestingly, the hyperthermophilic Bacterium *Thermotoga maritima* does not contain AOR, FOR, GAPOR, IOR, KGOR or VOR but uses POR (pyruvate ferredoxin oxidoreduc-

tase), ferredoxin and hydrogenase representing the lowest branches in the Bacterial line of descent supporting its thermophilic ancestor.

An interesting observation may be made with the uniqueness of these enzymes found in the Archaeal kingdom which have no counterpart in Bacterial or Eucaryal kingdom.

## Evolutionary development of redox proteins

The close environmental relationship between submarine hot springs and the Archaean period of the Earth has led to the proposal of the genetic take over and the mineral origins of life. The evolution of the Fe–S proteins from the sulphidic conditions of the primitive Earth and its ubiquitous presence in varied forms even today has uncovered Nature's attempt to chisel the formulae of these clusters with absolute fidelity. In continuation of Her search, She used FMDH during the initial steps of methane synthesis by methanogenic Archaea. Methane-producing microbes have been retrieved from depths of about 2.75 km where the temperatures run up to 75°C. These microbes apparently extract energy to convert dissolved $CO_2$ to biomass producing methane as a byproduct. These microbes grow on a diet of basaltic rocks that reacts with oxygen-free water to produce hydrogen – the only energy source in their ecosystem[26]. The initial reduction of $CO_2$ to formyl stage in methanogens involving the tungsten-containing enzyme FMDH and the involvement of other tungsten-dependent unique enzymes such as AOR, FOR and GAPOR of hyperthermophiles reflected Her choice to adopt thermostable redox systems to catalyse low potential reductions under anaerobic conditions ascertaining significant catalytic rates of these reactions only at high temperatures. Our current knowledge of the properties of these native tungsten enzymes[75] (molecular properties, Table 3) and prototungsten enzymes[76,77] justifies this scenario.

It is to be noted that moderate thermophily (< 80°C) is not a primitive but rather a derived character. This secondary thermophily may be acquired by genetic adaptation of mesophiles. The existence of several isoenzymes containing tungsten and molybdenum where the former exists in obligately anaerobic species in contrast to aerobic molybdenum-containing species is probably a relic of anoxic hyperthermophilic to oxic mesophilic via thermophilic regime of the geological time scale. The new qualities of the environment created by the gradual cooling of the Earth with anoxic to oxic transitions by the advent of algae allowed conservation of common carbon assimilation pathways. Thermophilic autotrophic anaerobe, *Halogenobacter*; microaerophilic *Aquifex*; microaerophilic heterotroph, *Thermotoga*; and the aerobic autotroph, *Hydrogenobacter* or the green sul-

Table 3. Molecular properties of tungstoproteins[44]

| Organism and enzyme | Holoenzyme molecular mass (kDa) | Sub-units | Sub-unit size (kDa) | W content | FeS or cluster content |
|---|---|---|---|---|---|
| Hyperthermophiles | | | | | |
| P. furiosus AOR | 136 | $\alpha_2$ | 67 | 2 | 2 [Fe$_4$S$_4$] + 1 Fe |
| P. furiosus FOR | 280 | $\alpha_4$ | 69 | 4 | 4 [Fe$_4$S$_4$] |
| P. furiosus GAPOR | 63 | $\alpha$ | 63 | 1 | ~ 6 Fe |
| T. litoralis FOR | 280 | $\alpha_4$ | 69 | 4 | 4 [Fe$_4$S$_4$] |
| Thermococcus sp. ES-1 AOR | 135 | $\alpha_2$ | 67 | 2 | 2 [Fe$_4$S$_4$] + 1 Fe ? |
| Pyrococcus sp. ES-4 AOR | 135 | $\alpha_2$ | 67 | 2 | 2 [Fe$_4$S$_4$] + 1 Fe ? |
| Methanogen | | | | | |
| M. thermoautotrophicum FMDH | 160 | $\alpha\beta\gamma\delta$ | 65, 53, 31, 15 | 1 | ~ 8 Fe |

phur Bacteria are the deep-rooted Bacterial lineage which conserve some common heritage of Archaeal domain[78].

## Closeness of Archaeal/Eucaryal domains

The Archaea and Eucarya likely had a common ancestor not shared by the Bacteria and the first organisms to have diverged from the Archaea/Eucarya lineage were the hyperthermophiles[2,79]. The similarities in the molecular machinaries of Archaea and Eucarya used in the first step of gene expression, the transcription of genes in RNA, furnished evidence to this lineage[80]. Researchers found that several Archaeal proteins involved in gene transcription resemble those of Eucarya more than those of Bacteria. For example, gene control sequences in Archaeal DNA resemble the TATA box, a regulatory sequence found in Eucarya but not in Bacterial genes. Even the Archaeal TBP (TATA binding protein) bears a remarkable resemblance to the human proteins; as their proteins are 40% identical[81]. Analysis of the deduced amino acid sequence for Pyrococcus furiosus DNA polymerase revealed that it had considerable homology with highly conserved regions in $\alpha$-like polymerases, a group that includes human, yeast, and other eucaryal DNA polymerases, but was much less homologous with Pol1-like DNA polymerases, which include the bacterial and Bacteriophage enzymes[82].

## Stability of the extremophiles

The stability of hyperthermophiles under extreme conditions can be related to the existence of life in an atmosphere similar to a pressure cooker. Quantitative thermodynamical studies demonstrate that proteins (enzymes) isolated from hyperthermophiles are inactive at 40°C, become fully activated at 90°C and unfold at 113°C. Thus 113°C seems to be the threshold tempera-

Table 4. Cloned and sequenced enzymes from sulphur-dependent Archaea[87]

| Organism/enzyme | Expression in E. coli |
|---|---|
| Pyrococcus furiosus | |
| Ferredoxin | Yes (purified) |
| DNA polymerase | Yes (purified) |
| Amylase | Yes (not purified) |
| Glutamate dehydrogenase | Yes (purified) |
| Histone proteins | No |
| Hydrogenase | No |
| Prolyl oligopeptidase | No |
| Maltose regulated gene | No |
| Pyrococcus woesei | |
| GAPDH | Yes (purified) |
| Glutamine synthetase | No |
| Thermococcus litoralis | |
| DNA polymerase | Yes (purified) |
| Pyrococcus strain ES-4 | |
| Glutamate dehydrogenase | Yes (not purified) |

ture of sustaination of life on Earth[83]. Evidences show that the organic solvents which are fatal for most common proteins proved to be perfect survival basis of these microorganisms. Horikoshi found that microbes for unknown reasons manage to thrive in toluene, benzene, cyclohexane and kerosene at almost a solvent concentration up to 50% (ref. 84).

Cloning and expression of genes encoding extremozymes from thermophilic Archaea have been started because of their many dimensional utilities on both scientific and technological fronts. Table 4 lists the enzymes which have been purified from both natural and recombinant sources. The high thermal stability of globular protein from Archaea is found to be an intrinsic property as manifested in recombinant proteins. The genes for the ferredoxin from P. furiosus when expressed in E. coli yield proteins that are as stable as the native form. Both recombinant and native P. furiosus

ferredoxin remain intact at 95°C for 24 h without significant denaturation[85]. Thus it is apparent that the information needed to confer extreme thermal stability is encoded in the gene sequences which do not require any extraneous factors. It has also been demonstrated that thermotolerance is an inducible state induced by a small cytoplasmic RNA (G8RNA). The gene encoding G8RNA is transcribed by RNA polymerase III (ref. 86). Many thermophilic enzymes are active enough in *E. coli* to produce toxic effects. The *Thermococcus litoralis* DNA polymerase intein-2 product, PI-TLiI, when expressed in *E. coli*, cuts the *E. coli* genome over 10 times which is much faster than expected for an endonuclease with a large and specific recognition sequence. Interestingly, it has been shown that PI-TLiI enzyme activity decreases drastically when the salt in the buffer is decreased. The reduced specificity of this enzyme at lower salt concentration is related to its toxicity in *E. coli*[87].

Heat-loving microbes seem to prevent thermal denaturation of their genetic material by twisting their DNA in the right-hand direction, leading to a positive supercoiled double-stranded chain. This unique topological conformation of DNA is generated by reverse gyrase, an ATP-dependent enzyme found only in hyperthermophilic organisms[88-93]. Kelly has found that a number of high temperature enzymes have a fewer of the amino acids most vulnerable to heat[94]. Adams suggested that some extremozymes use a 'salt bridge' – a zipper like series of ionic bonds through the interior that makes the structure difficult to unravel. Forterre and his associates have shown that reverse gyrase is a combination of a DNA helicase and a DNA topoisomerase[95]. Interpretation of their discovery is that reverse gyrase is the result of a gene fusion event that took place before the appearance of heat loving organisms but after the origin of life, once DNA genomes and the enzymes involved in their unwinding and replication had evolved. In addition to the hyperthermostability of hyperthermophiles, problem arises as to the involvement of various biological cofactors and intermediates which are extremely thermolabile. Some stabilization of these cofactors, like NADPH or NADH or intermediate organic substrates should occur *in vivo* for cellular function around 100°C. It has been observed in some cases that cytoplasmic solutes play a role in stabilizing these molecules[96-101] but no universal mechanism has yet been established.

Extremozymes are example of homeostatic systems. The study of structures of such enzymes will contribute immensely to modify some enzymes to withstand not only high temperatures, but environments even more hostile to life such as found in the organic solvents used in many industrial processes. What are the unique characteristics of the extremozymes that lead to their stability under very extreme conditions? This is one of the most puzzling and challenging problems, in both bio-

technology and biochemistry, which is yet to be resolved completely. One of the reasons is limited availability of structural data on proteins from the extremophiles.

Comparison of thermophiles with their mesophilic counterparts shows that there is not much difference in the sequence of the amino acids and it is hence very unlikely that these minor changes will result in tremendous increase in the thermal stability of a protein. In fact, change in stability is a consequence of some interactions that result in a global change. Hence sufficient and detailed information regarding three-dimensional structure of the protein is required. Proteins isolated from the anaerobic sulphur-reducing hyperthermophilic Archaeon *Pyrococcus furiosus* were studied. Two non-heme iron proteins rubredoxin (MW 5400) and ferredoxin (MW 7500) and the enzyme glutamate dehydrogenase (GDH) (MW 270,000) were studied for their thermostability. Independent of the particular amino acid sequence in the core, i. e. their primary structure, and independent of the molecular weight of the three proteins studied, the interactions within the hydrophobic core dwindle and finally vanish when the environmental temperature approaches 113°C, as a result of which all the three proteins investigated unfold at that very temperature irreversibly and irrespective of all the differences in size and sequence composition[83].

The detailed mechanism of the denaturation of proteins by temperature or in organic solvents, detergent, etc. is poorly understood but the evidence that thermostable proteins show enhanced resistance to all these agents implies that some aspect of the unfolding is common to all. Restriction of the initial reversible conformational transitions, a consequence of thermostabilization, will proportionately reduce the tendency of the protein to partake in future irreversible unfolding steps. The observation that chemical degradation of proteins, including the deamination of asparagine residues and peptide cleavage at Asn–Xaa bonds, proceeds more rapidly in pre-denatured proteins, also supports this mechanism[102,103].

Comparisons of the crystal structures of rubredoxin and ferredoxin with their mesophilic counterparts showed that the hydrophobic core of the hyperthermophilic proteins exhibited significant sequence and structural homology to the mesophilic proteins and had identical folding topologies. Hyperthermostability was achieved by relatively minor changes in the surface residues. These changes resulted in the modification of some secondary structural elements, such as a slightly longer helix, a triple rather than double-stranded β sheet and specific ionic interactions involving C and N terminal residues. For example, the N terminus is probably the first part of the protein to unzip at high temperatures and the enhanced stability of the *P. furiosus* rubredoxin protein likely results in part from a reduction in the la-

bility of its N terminus. The improved stability of rubredoxin can also result from improved main chain to main chain H-bonds and changes in buried surface area[83,87,104-106].

The enzyme aldehyde ferredoxin oxidoreductase (AOR) has no mesophilic equivalent. In specific comparisons with the structures of over thirty mesophiles, it has been shown that AOR contained a slightly increased number of ionic pairs per residue. AOR also exhibited minimum solvent exposed surface and a maximum fraction of buried atoms. In general, minimization of the ratio of surface area to volume increases the stability of an object by simultaneously reducing the unfavourable surface energy and increasing the attractive interior packing interactions[87,107]. It remains to be determined, however, if this is a general feature of hyperthermophilic enzymes or is specific to AOR.

Halophilic Archaea flourishing in hypersaline environment, where the water has 30 times the salinity of sea, such as the Dead Sea, the Great Salt Lake and the salt marshes, have developed certain mechanisms to overcome the extracellular osmotic pressure. Halophilic Archaea accumulates inorganic ions within the cell to concentrations exceeding that of the medium to balance the external high salt concentrations of their hypersaline medium. Therefore, the entire machinery of these organisms must be adapted to work under hypersaline environments. These organisms require high salinity for their active functioning and for their survival. Salt, like solvents, dehydrates enzymes[108,109].

The amino acid sequence of the enzyme halophilic malate dehydrogenase (hMDH) from the Archaean *Haloarcula marismortui* shows an excess of negatively-charged acidic groups over basic residues distributed on the enzyme surface and more salt bridges were present in it in comparison to its nonhalophilic congeners. Acidic residues, especially Glu, have the capability of binding more water than other residues which may lead to the creation of a hydration sphere that protects the enzyme from aggregating at high salt concentrations. Acidic residues also stabilize the folded native protein conformation by participation in an unusually large number of salt bridges. Other features that contribute to the stabilization of hMDH are the incorporation of alanine into $\alpha$ helices and the introduction of negatively-charged amino acids near their amino termini, both of which stabilize the $\alpha$ helix as a result of interaction with the positive part of the $\alpha$ helix dipole[103].

Clark found that enzymes from organisms living in deep sea vents, where boiling heat and crushing pressure combine, become more stable and more active when the pressure is increased. In addition to the salt bridges noted by Adams, the enzymes also have a densely-packed interior held together by water-excluding hydrophobic bonds. According to Clark, the enzymes that be-

come more stable under pressure are probably relying on the hydrophobic bonds for high temperature stability because pressure destabilizes salt bridges and stabilizes the hydrophobic bonds[109].

## Harvesting biodiversity

With the discovery of polymeric chain reaction (PCR) incorporating thermostable DNA polymerase from the thermophilic bacterium *Thermus aquaticus* (Taq), interest in this enzyme from hyperthermophiles has become substantial. Vent and deep vent polymerases and Pfu DNA polymerase, purified from *T. litoralis* strain GB-D and *P. furiosus* respectively are now commercially available. Details on the high fidelity of the *P. furiosus* DNA polymerase in the PCR reaction have been reported and both the *P. furiosus* and *T. litoralis* enzymes have been cloned and sequenced[82,110,111]. Recently the PCR technique has been improved to allow synthesis of long sequences (20–40 kB) by adding small amounts of thermostable proof reading DNA polymerases to Taq DNA polymerases[112,113]. Furthermore, thermophilic DNA ligases have been utilized in another technique called ligase chain reaction (LCR)[87].

Attempts have been made to know more about the thermophilic organisms by means of recreating evolution. Modern cool-water Bacteria differ from their thermophilic ancestors in many ways and it is very difficult to know which of these differences are there by random genetic drift and which are important. Petsko, with a speeded-up version of natural selection, attempted to make heat-loving organisms that are adapted to cooler temperatures. Using PCR technique he created a cold-water mutant version of an enzyme essential for synthesis of the amino acid leucine in Bacteria, the structure of which hints at the nature of the adaptation: Cold water turns the heat-adapted enzyme to an ineffective, rigid brick. The cold adapted mutant stays flexible[104].

Studies of enzyme systems from organisms inhabiting extreme environments are of interest not only for determining the physical limits of life on Earth but also for examining the mechanism by which organisms evolve specific biochemical and molecular adaptations. This evolutionary technique can be manipulated to design superior enzymes for industrial catalysis. Extremophiles thus helped the directed evolution of industrial enzymes. *S. acidocaldarius* is one of many bacteria investigated as being potentially useful for coal desulphurization but is so far unique in that the possible application of both its heterotrophic and chemolithotrophic metabolic activities have been raised[114]. The removal of inorganic sulphur fraction of coal through the Bacterial oxidation of pyrites ($FeS_2$) has been demonstrated numerous times in the laboratory but the problem of developing an economic process on the massive scale still remains formi-

dable[115]. Biorefining of diesel oil involves the use of unique enzymes and solvent-tolerant microbes. In biodesulphurization the oil phase can be extremely hostile to the biocatalyst and make access to the target molecules difficult. Since the sulphur molecules in diesel are present at low concentrations and are virtually insoluble in water, the use of extremophiles stable in organic solvents led to the development and metabolic engineering of whole-cell biocatalyst which operate in water:oil mixtures. Molecular chaperones are a class of proteins that are known to assist in folding some enzymes *in vitro* and have been suggested to be a means of enhancing the recovery of recombinant enzymes in industry. Research on hyperthermophilic chaperones has revealed that beyond protein folding, chaperonins have other important functions *in vivo* and these functions suggest new industrial applications for chaperonins. ThermoGen has been an isolating enzyme from thermophilic organisms for the use as industrial biocatalyst. Several esterases have been shown to be useful in the resolution of pharmaceutically important racemic mixtures. The four actinomycetes isolated from the deep sea hydrothermal vents and cold seep sites were found to belong to the genus *Streptomyces* but to differ from all 38 terrestrial *Streptomyces*. The extremophile *Sulfolobus acidocaldarius*, an Archaeon, has the capability of spontaneous genetic exchange[116].

Archaeal lipids characterized by novel chemical structures and molecular architectures represent a most promising tool for bioelectronics. The usage lies due to the factors like presence of high temperature, extreme pH and high ionic strength values, stable lipid layers in the membrane of extremophiles[117]. A considerable research effort in the area of microbially enhanced oil recovery (MEOR) has shown that it is promising and that a recovery biotechnology may be developed. Successful biochemical processes would have to be cost effective and have certain attributes, such as being able to produce emulsifying agents and acidify the media. Further, the microbes producing these conditions must be tolerant to high salt concentrations and be capable of metabolizing high molecular weight compounds. Since oil reservoirs are also subject to temperature and pressure variations, successful microbials should be able to adapt to such environments. Thus thermophilic microorganisms appear to be good candidates for MEOR[118]. A better understanding of heat shock responses of Archaeal organisms would facilitate their exploration in the biotechnological industries; for example in engineering cells that exhibit an improved ability to withstand or recover from stress[119].

## Future

It has been estimated that >99% of microbes in most habitats are unidentified, unculturable and their enzymes are therefore unknown. Mobilizing the evolutionary power of Nature and accessing the DNA probe technology would expand our knowledge of their enzymes. The exploration of poorly-characterized environments can be especially rewarding and extreme environments in particular offer the robust enzymes for protein engineering and directed evolution. The National Science Foundation, USA has created an opportunity to enhance knowledge about 'Life in Extreme Environments' through a highly interdisciplinary, integrated research programme for which special funding is marked in the financial year 1997. Such research will provide the basis to understand not only how life originated and evolved on Earth but also how life may thrive on other planets. The preliminary evidence for the past existence of living organisms on Mars has stimulated the recognition that the study of extreme environments on Earth, and the study of life they support, may be the most effective path towards detecting and understanding the life forms that may exist beyond our own planet. The recent Pathfinder mission to Mars is a forward step in this direction.

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# MEETINGS/SYMPOSIA/SEMINARS

**DAE Symposium on Recent Advances in Genetic Epidemiology and Population Monitoring**

Date: 23–26 March 1998
Place: Chennai

Topics include: Human genetics and genetic diseases; Genetic variation, chromosome and DNA polymorphism; Molecular epidemiology; Cancer and reproductive epidemiology; Adaptation, stress response and biological defence; Low-dose effects and risk analysis; Statistics as applicable to population monitoring and epidemiology.

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**The XV Asia Pacific Cancer Conference**

Date: 5–8 December 1999
Place: Chennai

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