Isocitrate dehydrogenase of the thermoacidophilic archaebacterium *Sulpholobus acidocaldarius*

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The thermoacidophilic archaebacterium, *Sulpholobus acidocaldarius,* has been found to possess both NAD- and NADP-dependent isocitrate dehydrogenase activities. Evidence is presented to suggest that both enzymic activities are functions of the same protein: NAD^+ and $NADP^+$ compete with each other for the enzyme and do so with K_i values equal to their K_m values; thermal inactivation results in the loss of both activities at the same rate and copurification was observed on gel filtration, ion-exchange chromatography and polyacrylamide gel electrophoresis. The evolutionary significance of this unique isocitrate dehydrogenase is discussed.

Archaebacteria Sulpholobus *Isocitrate dehydrogenase* *Evolution*

1. INTRODUCTION

Isocitrate dehydrogenase catalyses the oxidative decarboxylation of isocitrate to 2-oxoglutarate. Eukaryotes contain both NAD- and NADP-specific isocitrate dehydrogenases; the NAD-linked enzyme is confined to mitochondria and is allosterically regulated consistent with a controlling role in the energy-yielding function of the citric acid cycle [1,2]. However, most eubacteria possess only the NADP-isocitrate dehydrogenase [3] which, like the eukaryotic NADP-enzyme, is not allosterically controlled [1–3].

To date, there are no reports on the properties of isocitrate dehydrogenase(s) from archaebacteria, organisms which, it is proposed, represent a third primary line of evolutionary descent [4]. The relationship of the archaebacteria to the two other evolutionary lineages (eubacteria and eukaryotes) is not well defined although it has been suggested [5] that the nuclear genome may be of archaebacterial, rather than eubacterial or independent, origin. We have begun an investigation of citric acid cycle enzymes from archaebacteria [6] as it is observed that in eukaryotes and eubacteria several of these enzymes possess a diversity of structure and regulation which correlates with the organism's taxomonic status [3,7].

We report here that, like eukaryotes, the thermoacidophilic archaebacterium, *Sulpholobus acidocaldarius,* possesses both NAD- and NADP-isocitrate dehydrogenase activities but that, uniquely, the two activities are a function of the same enzyme molecule.

2. EXPERIMENTAL

All chemicals used were of analytical grade. *S. acidocaldarius* (DSM 639) was grown aerobically at 70°C (pH 2.0) in the basal salts medium described in [8] with the addition of yeast extract (1 g/l); the bacteria were harvested by centrifugation. Cells (0.2 g wet wt) were suspended in 1 ml of 50 mM Tris–HCl buffer (pH 8.0) containing 2 mM EDTA and a cell extract was prepared by sonication as in [9].

Isocitrate dehydrogenase was assayed at 55°C in 20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 10 mM $MgCl_2$, 0.2 mM $NADP^+$ (or 1.0 mM NAD^+) and 4.0 mM isocitrate. The reaction, in a final volume of 1 ml, was started with isocitrate after the addi-

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tion of enzyme, and the increase in A_{340} was followed with time.

Gel filtration was performed at 25°C on a column (2 × 33 cm) of Sephacryl S-200 (superfine grade) in 20 mM Tris–HCl (pH 8.0), containing 1 mM EDTA and 0.1 M KCl. The column was calibrated with standard proteins of known M_r values as in [9]. Ion-exchange chromatography was performed at 25°C on the Pharmacia fast protein liquid chromatography system using the Mono Q anion exchanger in 50 mM diethanolamine buffer (pH 8.8). Protein was eluted with a linear gradient of 0–1.0 M NaCl.

Discontinuous gel electrophoresis at pH 8.9 was performed as in [10] using 7.5% polyacrylamide gels and bromophenol blue as the marker dye. Isocitrate dehydrogenase was visualised by coupling the isocitrate-dependent reduction of $NAD(P)^+$ to diformazan production [11].

3. RESULTS

3.1. Coenzyme specificity

In extracts of *S. acidocaldarius,* both NAD- and NADP-linked isocitrate dehydrogenase activities were observed. The activities depended hyperbolically on the concentrations of NAD^+ [K_m^{app} = 3.4 (±0.5) mM] and of $NADP^+$ [K_m^{app} = 30 (±2) μM] as they did on isocitrate concentration [K_m^{app} = 10 (±0.5) μM for both NAD- and NADP-dependent activities]. The V_{max} values were similar: 0.13 (±0.03) μmol/min per mg protein for the NAD-enzyme and 0.10 (±0.02) μmol/min per mg protein for the NADP-enzyme. The same ratio of activities was observed with assays at 30°C.

With the widely differing K_m values it was important to demonstrate that the observed NAD-dependent enzymic activity was not due to a small amount (~1%) of $NADP^+$ in the NAD^+ solution and that the two apparently different activities were not the result of the same cofactor. Thus, neither pig heart nor *Acinetobacter lwoffi* NADP-specific isocitrate dehydrogenases showed any enzymic activity with high concentrations of NAD^+, indicating purity of the cofactor. In addition, prior incubation of the NAD^+ with alkaline phosphatase did not reduce the NAD-linked isocitrate dehydrogenase activity of *Sulpholobus* extracts whereas all

Table 1

Substrate competition between NAD^+ and $NADP^+$

Substrate	Observed enzyme activity	Theoretical enzyme activity	
		One enzyme	Two separate enzymes
6.0 mM NAD^+	0.53		
0.6 mM $NADP^+$	1.01		
6.0 mM NAD^+ + 0.6 mM $NADP^+$	0.94	0.99	1.54
1.0 mM NAD^+	0.19		
0.2 mM $NADP^+$	0.94		
1.0 mM NAD^+ + 0.2 mM $NADP^+$	0.91	0.94	1.13
3.0 mM NAD^+	0.44		
0.03 mM $NADP^+$	0.54		
3.0 mM NAD^+ + 0.03 mM $NADP^+$	0.59	0.66	0.98

Theoretical enzyme activity for one enzyme capable of utilizing both NAD^+ and $NADP^+$ is given by [14]:

$$V_{total} = \frac{V_{NAD}\left(1 + \frac{[NAD]}{K_m^{NAD}}\right) + V_{NADP}\left(1 + \frac{[NADP]}{K_m^{NADP}}\right)}{1 + \frac{[NAD]}{K_m^{NAD}} + \frac{[NADP]}{K_m^{NADP}}}$$

where V_{NAD} and V_{NADP} are enzyme velocities in NAD^+ or $NADP^+$ alone and the K_m values are those given in the text. Enzyme activity is in μmol product/min per ml

NADP-dependent activity was lost after similar treatment of the $NADP^+$. Finally, with limiting amounts of cofactor in the assays, the total changes in A_{340} for NAD^+ and $NADP^+$ were identical.

3.2. *Evidence for a single isocitrate dehydrogenase, with specifities for NAD^+ and $NADP^+$*

Assays of isocitrate dehydrogenase in extracts of *Sulpholobus* were carried out in NAD^+, $NADP^+$ and NAD^+ plus $NADP^+$ (table 1). The NAD- and NADP-linked enzymic activities were not additive indicating that they did not result from two independent enzymes. Rather, the data suggest that NAD^+ and $NADP^+$ compete with each other for the oxidation of isocitrate and do so with K_i values equal to their K_m values, i.e., they appear to bind at the same active site on a single isocitrate dehydrogenase. This suggestion was supported by thermal inactivation experiments. Extracts of *Sulpholobus* were incubated at 95°C and it was observed that the two enzymic activities were lost coincidently, the rate constants for the inactivations being 0.24 (±0.02) min^{-1} for the NAD-linked activity and 0.22 (±0.01) min^{-1} for the NADP-linked activity (fig.1). Further evidence for one enzymic protein was gained from polyacrylamide gel electrophoresis of *Sulpholobus* extracts followed by the specific staining for isocitrate dehydrogenase activity. A single enzyme band was observed for the isocitrate-dependent staining in the presence of NAD^+, $NADP^+$ and NAD^+ plus $NADP^+$ (fig.2). In all 3 gels the R_f of the band with respect to the bromophenol blue dye was 0.21 (±0.01). No staining was observed with either cofactor in the absence of isocitrate.

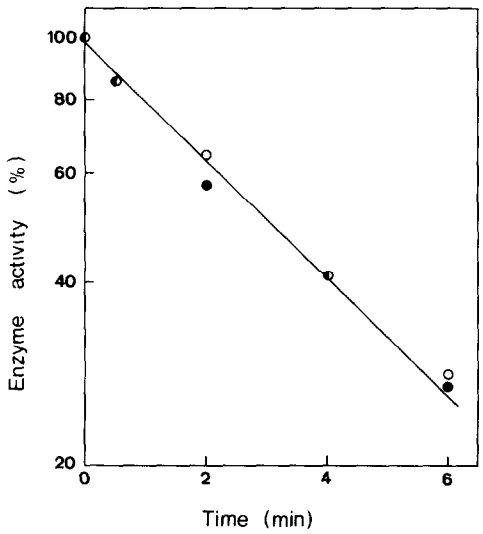

Fig.1. Thermal inactivation of isocitrate dehydrogenase. Extracts of *S. acidocaldarius* were incubated at 95°C and samples were removed at known time intervals, cooled to 4°C, and then assayed for NAD-linked (●), and NADP-linked (○) isocitrate dehydrogenase activities.

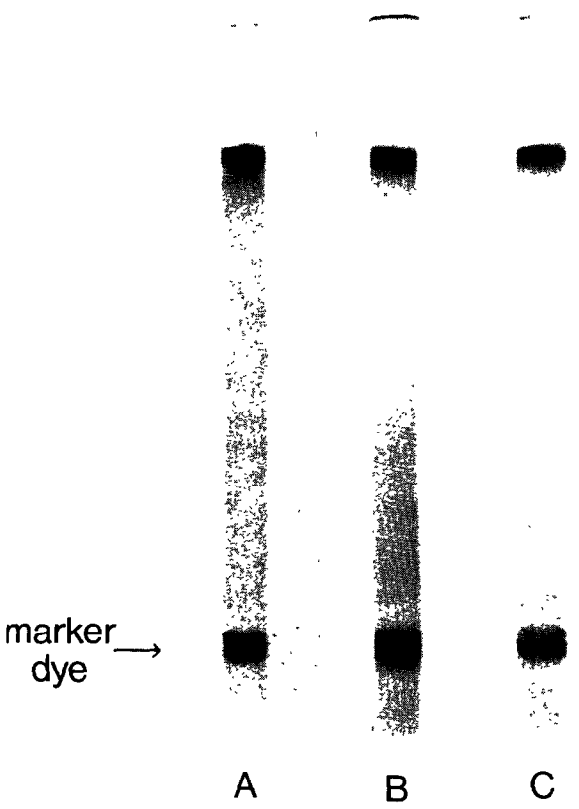

Fig.2. Polyacrylamide gel electrophoresis of extracts of *S. acidocaldarius*. Electrophoresis was performed as described in section 2, after which isocitrate dehydrogenase activity was visualised by coupling to diformazan production the isocitrate-dependent reduction of NAD^+ (gel A), $NADP^+$ (gel B) and NAD^+ plus $NADP^+$ (gel C).

Finally, copurification of the NAD- and NADP-linked isocitrate dehydrogenase activities was observed (table 2). From an extract of *Sulpholobus* the two activities co-eluted as a single, symmetrical peak on gel filtration through Sephacryl S-200. Analysis of the data with reference to standard proteins as in [9] gave an M_r for isocitrate dehydrogenase of 96000 (±4000). The fractions with the

Table 2

Purification of *Sulpholobus* isocitrate dehydrogenase

Step	Enzyme activity (V_{max}) (μmol/min)		Protein (mg)	Specific activity (μmol/min per mg)		Ratio of activities (NADP:NAD)	Yield (%)	
	$NADP^+$	NAD^+		$NADP^+$	NAD^+		$NADP^+$	NAD^+
Cell-free extract	1.75	1.22	18.0	0.10	0.07	1.4	100	100
Sephacryl S-200 pool	1.05	0.74	1.7	0.62	0.44	1.4	60	61
Pool from Mono Q ion-exchange chromatography	0.55	0.46	0.13	4.2	3.5	1.2	31	38

highest specific activities were pooled and chromatographed on an ion-exchange column as described in section 2. Again both NAD-and NADP-linked enzyme activities were co-eluted, this time in a single 1 ml fraction. The overall purification was > 40-fold.

4. DISCUSSION

The kinetic and structural evidence provided here suggests that the archaebacterium *Sulpholobus* possesses an isocitrate dehydrogenase which can use both NAD^+ and $NADP^+$. This contrasts with eukaryotes, where the two activities are functions of separate enzymes, and with eubacteria, the majority of which have only the NADP-linked dehydrogenase. However, in the light of speculations about the relationship between eukaryotic (nuclear) and archaebacterial genomes [5] some interesting comparisons can be made. First, for the *Sulpholobus* enzyme the K_m value for NAD^+ is approx. 100-times that for $NADP^+$. A similar ratio has been observed for the separate eukaryotic isocitrate dehydrogenases although the actual values of the constants are at least 10-fold smaller ([1] and refs therein). This difference could be the result of the temperature of assay (55°C) of the *Sulpholobus* isocitrate dehydrogenase, as it has been noted [12] that an increase in temperature produces a decrease in nucleotide binding affinity for some dehydrogenases. Secondly, it has been found [13] that pig heart NAD-linked isocitrate dehydrogenase also binds NADPH but probably at a different site to the NAD^+. Thus no catalytic activity is detectable with $NADP^+$ but like the enzyme from *Sulpholobus* it does bind both cofactors. The reciprocal situation of NAD(H) binding to the NADP-specific enzyme has not been reported.

The two eukaryotic isocitrate dehydrogenases exhibit considerable differences in their oligomeric nature; the NADP-enzyme is a monomeric protein [1] which may dimerise in the presence of substrates [1,2] whereas the NAD-linked dehydrogenase has 3 different subunits (α, β and γ) in the stoichiometry of 2:1:1 [1]. However, chemical modification studies indicate close similarities in the active site regions of the two enzymes [1] although the extent of homology awaits more detailed chemical studies.

Given the interest in the relationship between the different isocitrate dehydrogenases [1–3] it would appear important to examine in further detail the archaebacterial enzyme which combines the cofactor specificities.

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