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IspH Protein of *Escherichia coli*: Studies on Iron−Sulfur Cluster Implementation and Catalysis

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Abstract: The *ispH* gene of *Escherichia coli* specifies an enzyme catalyzing the conversion of 1-hydroxy-2-methyl-2-(*E*)-butenyl diphosphate into a mixture of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) in the nonmevalonate isoprenoid biosynthesis pathway. The implementation of a gene cassette directing the overexpression of the *isc* operon involved in the assembly of iron−sulfur clusters into an *Escherichia coli* strain engineered for *ispH* gene expression increased the catalytic activity of IspH protein anaerobically purified from this strain by a factor of at least 200. For maximum catalytic activity, flavodoxin and flavodoxin reductase were required in molar concentrations of 40 and 12 μM, respectively. EPR experiments as well as optical absorbance indicate the presence of a $[3Fe\text{-}4S]^{+}$ cluster in IspH protein. Among 4 cysteines in total, the 36 kDa protein carries 3 absolutely conserved cysteine residues at the amino acid positions 12, 96, and 197. Replacement of any of the conserved cysteine residues reduced the catalytic activity by a factor of more than 70 000.

Introduction

The rapid spreading of resistance against all currently used anti-infective agents in pathogenic bacteria, fungi, and protozoa is one of the most dramatic, but still widely underestimated medical problems worldwide. Without the rapid deployment of novel anti-infective agents, a rapid and massive increase in morbidity and mortality due to infective disease is imminent not only in the developing world, but also in industrialized countries.[1−4] This could reverse the steady increase in life expectancy that has occurred over the past decades[5] and result in major socioeconomic changes of a global nature. However, the development of novel antiinfective drugs is presently not a high priority of major pharmaceutical companies.[6]

The majority of presently used antibiotics interfere with the biosynthesis of macromolecular constituents of microbial pathogens.[7] More specifically, important groups of therapeutic agents affect DNA replication, transcription, and translation.[8] By comparison, the biosynthesis of low molecular weight compounds plays only a limited role in antimicrobial and antiparasite strategies. A notable exception is the biosynthesis of tetrahydrofolic acid;[9] in fact, the sulfonamides which inhibit dihydropteroate synthase were the first antimicrobial drugs with a broad action spectrum that reached the market.

A likely reason for the success of tetrahydrofolate biosynthesis inhibitors is the absence, in the respective pathogens, of transport systems for the uptake of tetrahydrofolate and its derivatives from the environment. A similarly favorable situation exists in case of terpenoid biosynthesis where certain essential products of the highly pleiotropic pathway cannot be acquired from the environment.[10]

In most pathogenic bacteria (with the exception of Gram-positive cocci), in apicomplexan parasites (most notably the genus *Plasmodium*) and in the plastids of plants the precursors for the synthesis of essential terpenoid metabolites are supplied by the recently discovered nonmevalonate isoprenoid biosyn-

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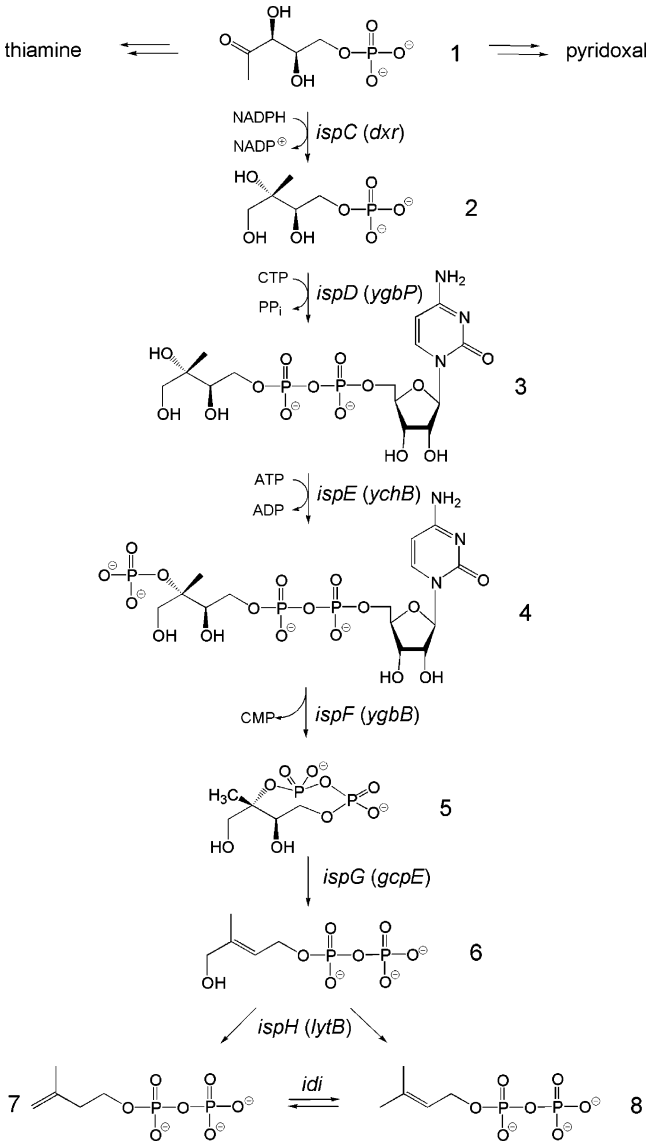
thesis pathway.[11−17] This pathway has been validated as a target for the therapy of malaria.[18,19] Since animals use the mevalonate pathway for the biosynthesis of terpenoids, inhibitors of the nonmevalonate pathway should not interfere with mammalian terpenoid biosynthesis.

The first committed intermediate in the nonmevalonate pathway is the branched chain polyol derivative, 2*C*-methyl-D-erythritol 4-phosphate (**2**), which is obtained from 1-deoxy-D-xylulose 5-phosphate (**1**) by isomerization and reduction[20,21] and has also been shown to serve as precursor for the biosynthesis of the vitamins B_1 and B_6 (Scheme 1).[22−25] 2*C*-methyl-D-erythritol 4-phosphate (**2**) is converted into the cyclic diphosphate **5** by a series of three reactions catalyzed by enzymes specified by the *ispDEF* genes.[26−28] The cyclic diphosphate **5** is converted into 1-hydroxy-2-methyl-(*E*)-butenyl 4-diphosphate (**6**) by a mechanistically complex reaction which is catalyzed by IspG protein in cooperation with incompletely characterized redox cofactors.[29−32]

The product **6** of IspG protein is converted into a mixture of isopentenyl diphosphate (IPP) (**7**) and dimethylallyl diphosphate (DMAPP) (**8**) by IspH protein.[33−36] Under in vitro conditions,

Scheme 1. The Deoxyxylulose Phosphate Pathway



reduction equivalents for that reaction can be provided by dithionite,[33] photoreduced deazaflavin[32,35,36] or a mixture of flavodoxin, flavodoxin reductase, NAD(P)H and FMN or FAD.[32,35,36] Depending on the nature of the various electron donors, the catalytic rates reported in the literature differ by several orders of magnitude.

IspH protein has been shown to include an iron−sulfur cluster which suggests a radical mechanism.[32,33,35,36] In this paper, we show that the recombinant expression of the protein in functional form can be greatly enhanced by coexpression of genes from the *isc* operon which are required for the assembly of iron−sulfur clusters.[37]

Experimental Section

Materials. A sample of **6** was prepared as described earlier.[38] [1-^{3}H]-**6** (4.2 mCi μmol^{-1}) was synthesized by published procedures.[39] [3,4-$^{13}C_2$]-**6** was prepared by adaptation of the published synthesis[38]

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Table 1. Bacterial Strains and Plasmids Used in This Study

strain or plasmid	genotype or relevant characteristic	ref. or source
E. coli		
XL1-Blue	*RecA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac,* [F', *proAB, lacI*^{q}*ZΔM15, Tn10 (tet*^{r})]	stratagene[41]
plasmids		
pQElytB	hyperexpression of *ispH* (*lytB)* from *E. coli* as N-terminal His-taq protein	34
pACYC184	low copy cloning vector	NEB[40]
pACYCisc	expression of *the isc operon* from *E. coli*	this study

Table 2. Oligonucleotides Used in This Study

designation	5′-sequence-3′
iscSBamHIvo	CAGGTTGGATCCGAGAGAGAAATTAACC-ATGTACGGAGTTTATAGAGC
fdxSalIhi	CCAGTAGTCGACTTAATGCTCACGCGCATGG
lytBvo	TGGAGGGGATCCATGCAGATCCTGTTGGCC
lytBhi	GCATTTCTGCAGAACTTAGGC
LytBC12SRev	GTCTACCCCGGC*AGA*AAAACCACGCG
LytBC12SFw	CGCGTGGTTTTT*TCT*GCCGGGGTAGACC
LytBC96SRev	GGTCACCAGCGG*AGA*GGTGGCGTCG
LytBC96SFw	GGTCACCAGCGG*AG*AGGTGGCGTCG
LytBC197SRev	CGTGGCGTA*AGA*GATGTCATCTTTGCG
LytBC197SFw	CGCAAAGATGACA*TCT*CTTACGCCACG

Mutated nucleotides are shown in italic.

using the ethylester of [1,2-$^{13}C_2$]bromoacetic acid as the labeled starting material. Oligonucleotides were custom synthesized by MWG Biotech (Ebersberg, Germany). Type I IPP isomerase (Idi-1 protein) was prepared according to published procedures.[32] 10-Methyl-5-deazaisoalloxazine (deazaflavin) was a generous gift of Prof. Andrée Marquet, Paris.

Microorganisms. Bacterial strains and plasmids used in this paper are summarized in Table 1.

Construction of a Recombinant *E. coli* Strain Coexpressing *ispH* and the *isc* Operon. A part of the *isc* operon from *E. coli* was amplified with the oligonucleotides iscSBamHIvo and fdxSalIhi (Table 2) by PCR from base pair 2,654,768 through 2,659,551 using the high fidelity DNA polymerase DyNAzyme (Finnzymes, Espoo, Finland) and chromosomal *E. coli* DNA as template (Figure 1). The amplificate was digested with *BamH*I and *Sal*I and was then ligated into the plasmid pACYC184[40] which had been treated with the same restriction endonucleases. The resulting plasmid pACYCisc was transformed into *E. coli* strain XL1-Blue (Stratagene).[41] The plasmid was reisolated and transformed into *E. coli* strain XL1-Blue harboring the plasmid pQElytB which directs the expression of recombinant *ispH* gene of *E. coli* with an N-terminal polyhistidine tag under the control of a T_5 promoter and *lac* operator.[34] The strain was cultured in LB medium containing chloramphenicol (25 mg l^{-1}) and ampicillin (180 mg l^{-1}) to enforce the maintenance of both plasmids.

Site-Directed Mutagenesis. Site-directed mutagenesis was performed by PCR using the overlap extension technique.[42] PCR was performed with Dynazyme II DNA polymerase (Finnzymes). The internal mismatch primers are shown in Table 2. Mutations are underlined.

The general scheme of mutagenic PCR involved three rounds of amplification cycles using two mismatch and two flanking primers (primers lytBvo and lytBhi, Table 2). During the first round, 20 amplification cycles were carried out with one of the flanking primers and the corresponding mismatch primer. The plasmid pQElytB was used as template. During the second amplification cycle, 20 amplification cycles were carried out using the second flanking primer and the corresponding mismatch primer. The plasmid pQElytB was used as template. Amplificates were purified by agarose gel electrophoresis and isolated using the EZNA Gel Extraction kit (Peqlab, Erlangen, Germany). During the third round, the products of round one and two were used as templates, and 20 amplification cycles were carried out using the two flanking primers. The resulting amplificate was subjected to agarose gel electrophoresis, digested with *Bam*HI and *Pst*I, purified using the EZNA Cycle pure purification kit (Peqlab, Erlangen, Germany) and ligated into the plasmid pQE30 (Quiagen, Hilden, Germany) which had been digested with the same restriction enzymes. The ligation mixture was transformed into *E. coli* XL1-blue cells (Stratagene, Heidelberg, Germany). All gene constructs were verified by DNA sequencing (GATC Biotech AG, Konstanz, Germany).

Culturing Recombinant Bacteria. Recombinant *E. coli* strains were grown in Luria Bertani broth containing ampicillin (180 mg l^{-1}) and chloramphenicol (25 mg l^{-1}) as appropriate. Cultures were incubated at 37 °C with shaking. At an optical density of 0.7 (600 nm), isopropylthiogalactoside was added to a final concentration of 2 mM, and the cultures were incubated for 5 h. The cells were harvested by centrifugation, washed with 0.9% (w/v) sodium chloride, and stored at −20 °C under anaerobic conditions.

Preparation of Recombinant Proteins (FldA, Fpr, and IspH). All steps were carried out under anaerobic conditions in a glovebox. Frozen cell mass (25 g) from the recombinant *E. coli* strains XL1-pQEfldA,[32] XL1-pQEfpr,[32] or XL1-pQElytB-pACYCisc was thawed in 120 mL of 100 mM Tris hydrochloride, pH 8.0, containing 0.5 M sodium chloride and 20 mM imidazole hydrochloride. The cells were disrupted by treatment with a French Press, and the suspension was centrifuged. The supernatant was applied to a column of Ni-chelating Sepharose FF (Amersham Pharmacia Biotech; column volume, 20 mL) which had been equilibrated with 100 mM Tris hydrochloride, pH 8.0, containing 0.5 M sodium chloride and 20 mM imidazole (flow rate, 2 mL min^{-1}). The column was washed with 100 mL of 100 mM Tris hydrochloride, pH 8.0, containing 0.5 M sodium chloride and 20 mM imidazole and was then developed with a gradient of 20−500 mM imidazole in 100 mM Tris hydrochloride, pH 8.0, containing 0.5 M sodium chloride (total volume, 100 mL). Fractions were combined and dialyzed overnight against 100 mM Tris hydrochloride, pH 8.0 and stored at −80 °C.

Two-Dimensional Polyacrylamide Gel Electrophoresis. Experiments were performed according to Görg et al.[43] For identification, spots were excised and digested following the protocol of Schäfer et al.[44] Samples for MALDI-TOF−MS were prepared using ZipTips (Qiagen, Hilden, Germany) following the manufacturer's protocol. The resulting peptides were identified with a Biflex-II MALDI-TOF spectrometer (Bruker Daltonik, Bremen, Germany). Data analysis was performed with the MASCOT software server (Matrix Science, London, UK; http://www.matrix-science.com).

Determination of Iron. The iron content of as-isolated protein samples was determined according to the procedure of Brumby and Massey, 1967.[45]

Radiochemical Assay of IspH Protein. All steps were carried out under anaerobic conditions in a glovebox. Assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 30 mM sodium fluoride, 2 mM

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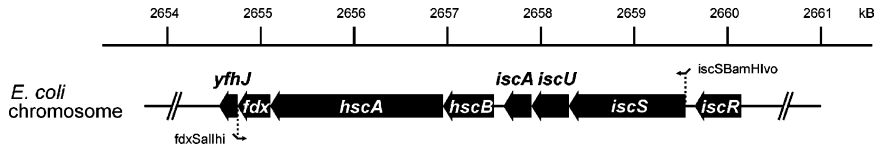

Figure 1. The *isc* operon of *Escherichia coli***.** Oligonucleotides used for PCR amplification and cloning are indicated by arrows. For details see Experimental Section.

NADPH, 2.3 μM [1-^{3}H]-**6** (4.2 mCi μmol^{-1}), 40 μM flavodoxin, 12 μM flavodoxin reductase, and IspH protein in a volume of 160 μL. The mixtures were incubated at 37 °C. The reaction was terminated by the addition of 5 μL of 3 M trichloroacetic acid, and the mixtures were centrifuged. The supernatants were neutralized with 5 μL of 3 M sodium hydroxide and were subjected to ultrafiltration (Nanosep 100 kDa membrane). Aliquots (50 μL) were analyzed by reversed-phase ion pair HPLC using a Luna C8 column (5 μm, 4.6 × 250 mm, Phenomenex, Aschaffenburg, Germany), which was washed with 10 mL of 3.5% (v/v) methanol in 10 mM tetra-*n*-butylammonium phosphate, pH 6.0 and then was developed with consecutive gradients of 3.5−21% (v/v) methanol (2 mL), 21−35% (v/v) methanol (13 mL) and 35−49% (v/v) methanol (10 mL) containing 10 mM tetra-*n*-butylammonium phosphate, pH 6.0 (flow rate, 1 mL min^{-1}). The effluent was monitored by realtime liquid scintillation analysis (Beta-RAM, Biostep GmbH, Jahnsdorf, Germany). The retention volume of **6** was 24 mL; **7** and **8** were jointly eluted at 36 mL.

Photometrical Assay of IspH Protein. All steps were carried out under anaerobic conditions in a glovebox. Assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 400 μM **6**, 30 mM sodium fluoride, 1 mM NADPH, 40 μM flavodoxin, 12 μM flavodoxin reductase, and IspH protein in a volume of 450 μL. The mixtures were incubated at 37 °C. The reaction was monitored photometrically at 340 nm.

^{13}C NMR Assay of IspH Protein (Method A). All steps were carried out under anaerobic conditions in a glovebox. Assay mixtures contained 40 mM Tris hydrochloride, pH 8.0, 30 mM sodium fluoride, 2 mM NADPH, 2 mM [3,4-$^{13}C_2$]-**6**, 40 μM flavodoxin, 12 μM flavodoxin reductase, and 16 μg IspH protein in a volume of 500 μL. The mixture was incubated at 37 °C. D_2O was added to a final concentration of 5% (v/v). The solution was analyzed by ^{13}C NMR spectroscopy.

(Method B). All steps were carried out under anaerobic conditions in a glovebox. Assay mixtures contained 50 mM Tris hydrochloride, pH 8.0, 7.5 mM dithiothreitol, 2 mM [3,4-$^{13}C_2$]-**6**, 1.4 mM deazaflavin (dissolved in dimethyl sulfoxide) and 16 μg IspH protein in a volume of 500 μL. The mixture was irradiated with a 100 W mercury vapor lamp (Osram) placed at a distance of 15 cm. During irradiation, the mixture was cooled on ice. D_2O was added to a final concentration of 5% (v/v). The solution was analyzed by ^{13}C NMR spectroscopy

NMR Spectroscopy. ^{13}C and ^{1}H NMR spectra were measured with a DXR 500 AVANCE spectrometer from Bruker Instruments, Karlsruhe, Germany.

EPR Sample Preparation. IspH protein samples (protein concentration about 12 mg mL^{-1}) were transferred into EPR suprasil quartz tubes (3 mm inner diameter) under an argon inert gas atmosphere and rapidly frozen in liquid nitrogen.

EPR Spectroscopy. X-band pulsed EPR spectra were recorded using a Fourier transform EPR spectrometer (Bruker ESP 580E) and a dielectric-ring resonator (Bruker ER4118-MD-5W1-EN), which was immersed in a helium-gas flow cryostat (Oxford CF-935). All experiments were performed at 5.5 K, and the temperature (deviation of ±1 K) was regulated by a temperature controller (Oxford ITC-4). For magnetic-field swept electron-spin−echo-detected EPR spectra, a microwave pulse-sequence $\pi/2-\tau-\pi$ using 32 and 64 ns $\pi/2$- and π-pulses, respectively, was used. The pulse-separation time τ between the microwave pulses was selected to be 200 ns. To avoid saturation effects, the entire pulse pattern was repeated with a frequency of 1 kHz. For a comparison with EPR spectra of other protein systems with iron−sulfur clusters, typically obtained by continuous-wave EPR, first-derivative EPR spectra with respect to the magnetic field were calculated from the pulsed EPR spectra using the program package XEPR (version 2.2) from Bruker. EPR spectral simulations were performed using the computer program SIMPOW6 obtained from Mark J. Nilges and the Illionios EPR research center (http://ierc.scs.uiuc.edu/nilges.html). The program generates an EPR powder spectrum for an $S = 1/2$ electron spin with a completely anisotropic g-matrix and an orientation-dependent spectral line width.

Results

We have previously reported a recombinant IspH protein of *E. coli* fused to maltose binding protein which catalyzed the conversion of **6** into a mixture of **7** and **8** at a rate of 3 nmol min^{-1} mg^{-1} in the presence of flavodoxin reductase, flavodoxin and NADH, whereas a higher catalytic rate (0.4 μmol min^{-1} mg^{-1}) was observed with photoactivated deazaflavin as reducing agent.[32] The absorption spectrum of the recombinant IspH protein suggested the presence of an iron−sulfur cluster which has been proposed to be crucial for catalytic activity.[32,33,36]

In an attempt to improve the specific activity of the native protein, we decided to construct an *ispH* hyperexpression strain endowed with a plasmid directing the hyperexpression of *isc* operon genes which are known to be involved in the assembly of iron−sulfur clusters.[37] A segment of the *E. coli isc* operon comprising the *iscS*, *iscU*, *iscA*, *hscB*, *hscA*, and *fdx* genes (Figure 1)[46] was amplified by PCR using a high fidelity DNA polymerase and was placed in-frame into the tetracycline resistance cassette of the low copy plasmid pACYC184[40] which is compatible with plasmids of the Col-E1 group due to its P15A replicon. This plasmid construct was transformed into the recombinant strain engineered for hyperexpression of a recombinant *E. coli* IspH protein with an N-terminal polyhistidine tag. Two-dimensional polyacrylamide gel electrophoresis in conjunction with mass spectrometric protein spot assignment confirmed that the genes *iscS*, *iscU*, *hscA*, and *ispH* were all significantly overexpressed by comparison with an *E. coli* strain harboring the vacant pACYC184 vector without insert (Figure 2; cf. Supporting Information for physical properties of Isc proteins).

Cell extracts of the *E. coli* strain expressing the *ispH* gene and the *isc* cluster genes catalyzed the conversion of **6** into a mixture of **7** and **8** at a rate of 62 nmol mg^{-1} min^{-1} under anaerobic conditions using a mixture of NADPH, flavodoxin reductase, and flavodoxin in order to supply reduction equivalents (Table 3). By comparison, cell extract of an *E. coli* strain which carries the plasmid for expression of the recombinant *ispH* gene but not that for the *isc* operon expression had only a catalytic activity of 2.8 nmol mg^{-1} min^{-1}. Thus, the apparent catalytic activity had been enhanced by a factor of at least 20

(46) Tokumoto, U.; Nomura, S.; Minami, Y.; Mihara, H.; Kato, S. I.; Kurihara, T.; Esaki, N.; Kanazawa, H.; Matsubara, H.; Takahashi, Y. *J. Biochem.* **2002**, *131*, 713−719.

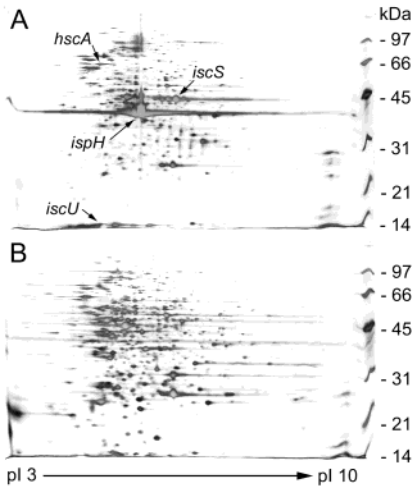

Figure 2. 2D gel electrophoresis. A, cell extract from a recombinant *E. coli* strain expressing the *ispH* gene and the *isc* operon from *Escherichia coli*. B, cell extract from *E. coli* with vacant vector**.** The spots of the proteins IscS, IscU, HscA, and IspH were identified by mass fingerprinting and are indicated by arrows. For details see Experimental Section.

Table 3. Purification of Recombinant IspH Protein from *Escherichia Coli*

procedure	total protein (mg)	specific activity (nmol min^{-1} mg^{-1})	total activity (nmol min^{-1})	yield (%)	purification factor
cell extract	3000	62	186 000	100	1
Ni^{2+}−Sepharose FF	200	700	140 000	75	11

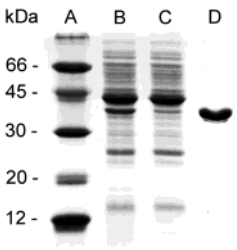

Figure 3. SDS polyacrylamide gel electrophoresis. Lane A, molecular weight markers; lane B, cell extract of a recombinant *E. coli* strain expressing the *ispH* gene and the *isc* operon from *Escherichia coli*; lane C, flow through from affinity chromatography on nickel chelating Sepharose FF of crude cell extract of recombinant *E. coli* cells expressing IspH protein; lane D, recombinant IspH protein after affinity chromatography on nickel chelating Sepharose FF.

by implementation of the *isc* operon genes, even though the expression of the *isc* operon genes had reduced the level of IspH protein expression (Figure 3, lane B).

The recombinant, histidine-tagged IspH protein could be purified 11-fold under anaerobic conditions by affinity chromatography on nickel chelating resin (Table 3). The 36 kDa protein (subsequently designated "as-isolated protein") migrated on the column as an intensely brown-colored band and appeared homogeneous after elution as judged by SDS polyacrylamide gel electrophoresis (Figure 3, lane D). The absorption spectrum showed a maximum at about 410 nm with a molar absorbance of 11 800 M^{-1} cm^{-1} and a shoulder at about 320 nm (Figure 4). The ratio of E_{410}/E_{280} was 0.38. The protein was found to contain 2.6 iron ions per subunit using the assay method of Brumby and Massey.[45]

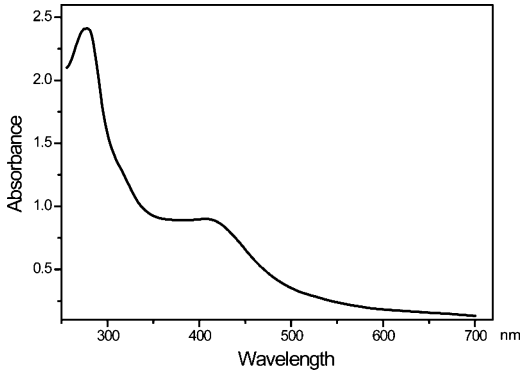

Figure 4. UV−vis spectrum of recombinant IspH protein. The protein concentration was 2.8 mg ml^{-1}. The spectrum shows a maximum at 410 nm and a shoulder at 320, indicating the presence of an iron−sulfur cluster.

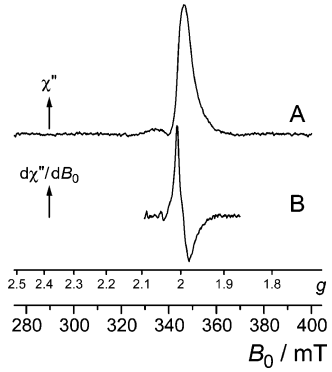

Figure 5. Pulsed EPR spectra of IspH protein. (A) Field-swept electron-spin−echo-detected EPR signal. (B) First-derivative EPR signal obtained by pseudo-modulation (1 mT modulation amplitude) of the field-swept EPR signal. Microwave frequency: 9.659 GHz.

To confirm the presence of an iron−sulfur center in the IspH protein, X-band EPR spectroscopy was applied. At 5.5 K, the protein gave a pulsed EPR signal (signal amplitude χ" as a function of the magnetic field B_0) centered approximately at 345 mT with a spectral line width of about 10 mT (see Figure 5, trace A). With the exception of a background signal arising from the EPR resonator, which was subtracted for further spectral analysis, no other EPR signals were observed within the spectral range from 280 to 400 mT. For better comparison with EPR spectra from protein-bound iron−sulfur centers reported in the literature, the first derivative of trace A (signal amplitude $d\chi"/dB_0$ as a function of the magnetic field B_0) is also shown in Figure 5, trace B. The spectrum with a zero-crossing at 346.1 mT is clearly asymmetric due to an anisotropic g-matrix. It consists of a narrow peak at 343.5 mT and a broader trough at 348.6 mT. The *g*-principal values of the spectrum obtained by a least-squares fitting routine reflect axial symmetry of the *g*-matrix: $g_{||} = 2.032(3)$, $g_{\perp} = 2.003(3)$. This is consistent with the presence of a $[3Fe\text{-}4S]^{+}$ cluster.

Enzymatic activity of the purified recombinant IspH protein was measured with synthetic [1-^{3}H]-**6** as substrate. Assay mixtures were analyzed by reversed-phase ion-pair HPLC, and product formation was monitored by on-line liquid scintillation counting (Figure 6). Results obtained with NADH, NADPH and with various redox shuttle proteins added in different combinations are shown in Table 4. Maximum catalytic activity of 0.7 μmol min^{-1} mg^{-1} was obtained with NADPH as cosubstrate, together with recombinant flavodoxin and flavodoxin reductase

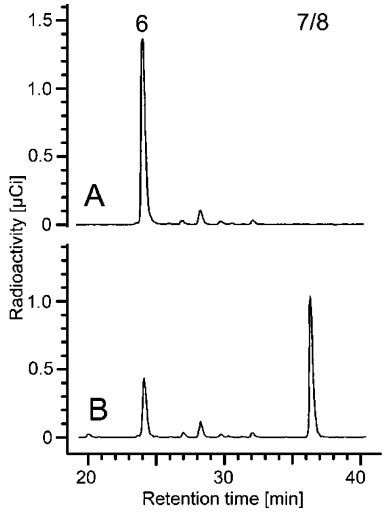

Figure 6. Enzymatic conversion of ^{3}H-labeled **6** into **7**/**8**. Assay mixtures were prepared as described in the Experimental Section. The concentrations of **7**/**8** and **6** were analyzed by reversed phase ion-pair HPLC monitored by on-line liquid scintillation detection. (A) Control sample before incubation. (B) Sample incubated for 10 min at 37 °C. The retention times of **6** and **7**/**8** are 24 and 36 min, respectively.

Table 4. Relative Catalytic Activities of Recombinant IspH Protein under Various conditions

supplements								relative activity (%)
IspH	FldA[a]	Fdx[b]	Fpr[c]	Fpr[d]	NADPH	NADH	NaF	
+	+	−	+	−	+	−	+	100
+	+	−	+	−	−	+	+	70
+	+	−	−	+	+	−	+	75
+	+	+	+	−	+	−	+	4.4
+	−	+	−	+	+	−	+	4.5
+	+	−	+	−	+	−	−	55
+	+	−	−	−	+	−	+	<0.0015
+	-	−	+	−	+	−	+	<0.0015

The reaction mixtures contained 100 mM Tris hydrochloride, pH 8.0, 2.3 μM [1-^{3}H]-**6** (4.2 mCi μmol^{-1}), and recombinant IspH protein from *E. coli* in a volume of 160 μL and were incubated and analyzed as described in the Experimental Section. [a] 40 μM recombinant flavodoxin from *E. coli*. [b] 4 μM ferredoxin from spinach. [c] 12 μM recombinant flavodoxin reductase from *E. coli*. [d] 0.76 μM ferredoxin reductase from spinach.

from *E. coli*. At low molar concentrations of flavodoxin and flavodoxin reductase, the apparent IspH activity increased linearly with the concentrations of these auxiliary proteins (Figure 7); saturation was observed with molar concentrations of flavodoxin and flavodoxin reductase of 40 and 12 μM, respectively. Notably, a mixture of ferredoxin and ferredoxin reductase from spinach could also serve as auxiliary proteins (cf. Table 4). The addition of divalent cations is not required for IspH activity. On the contrary, the addition of Ca^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}[47], and Fe^{2+} decreased IspH activity, and the addition of Zn^{2+} resulted in complete loss of activity (Supporting Information).

IspH activity could also be monitored by photometric observation (340 nm) of NADPH dehydrogenation. This assay method afforded a value of 0.55 μmol min^{-1} mg^{-1}. The Michaelis constant for the substrate **6** had a value of 30 μM (Figure 8). In contrast to studies on the homologous IspH protein from the thermophile *Aquifex aeolicus* described by Altincicek et al.,[33] we were unable to detect any enzymatic activity using dithionite as reducing agent in assays with the recombinant *E. coli* enzyme.

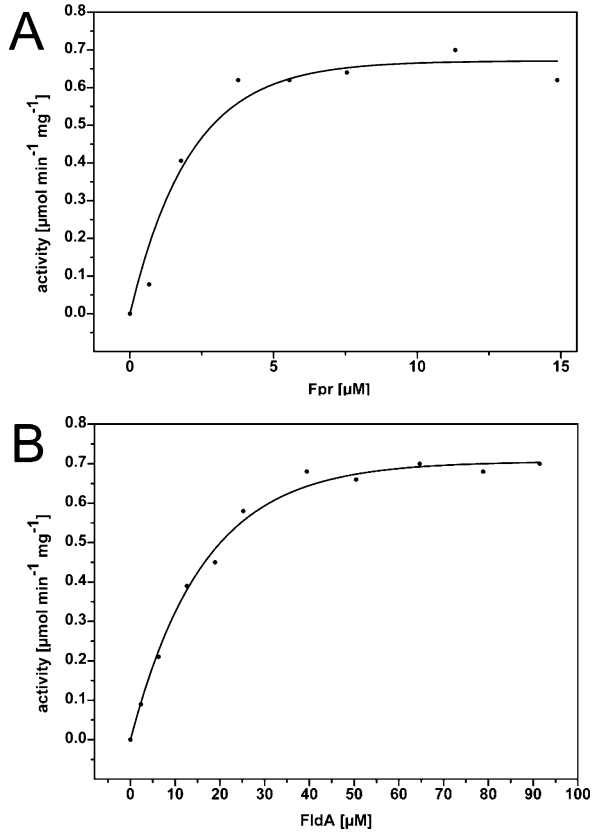

Figure 7. Activation of IspH protein by (A) flavodoxin reductase and (B) flavodoxin. The assay mixtures contained 100 mM Tris hydrochloride, pH 8.0, 30 mM sodium fluoride, 2 mM NADPH, 2.3 μM [1-^{3}H]-**6** (4.2 mCi μmol^{-1}), flavodoxin, flavodoxin reductase, and IspH protein in a volume of 160 μL and were incubated and analyzed as described in the Experimental Section. The constant concentrations of flavodoxin and flavodoxin reductase were 40 and 12 μM, respectively.

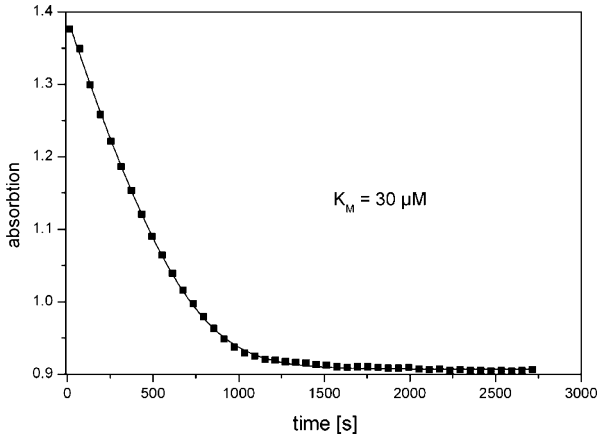

Figure 8. Kinetics of IspH protein activity. For details see Experimental Section. (■), absorbance at 340 nm (experimental data). The line represents the absorbance at 340 nm obtained from numerical simulation using the program biokin.[59]

IspH protein has been shown earlier to produce a mixture of **7** and **8**. To detect these products selectively, the enzyme-catalyzed reaction was monitored by ^{13}C NMR spectroscopy. To enhance the sensitivity and selectivity of ^{13}C NMR detection, we used [3,4-$^{13}C_2$]-**6** as substrate. The ^{13}C NMR signals of the substrate **6** (Figure 9A) and the products **7** and **8** obtained in this experiment (Figure 9B) all appear as doublets due to $^{13}C^{13}C$

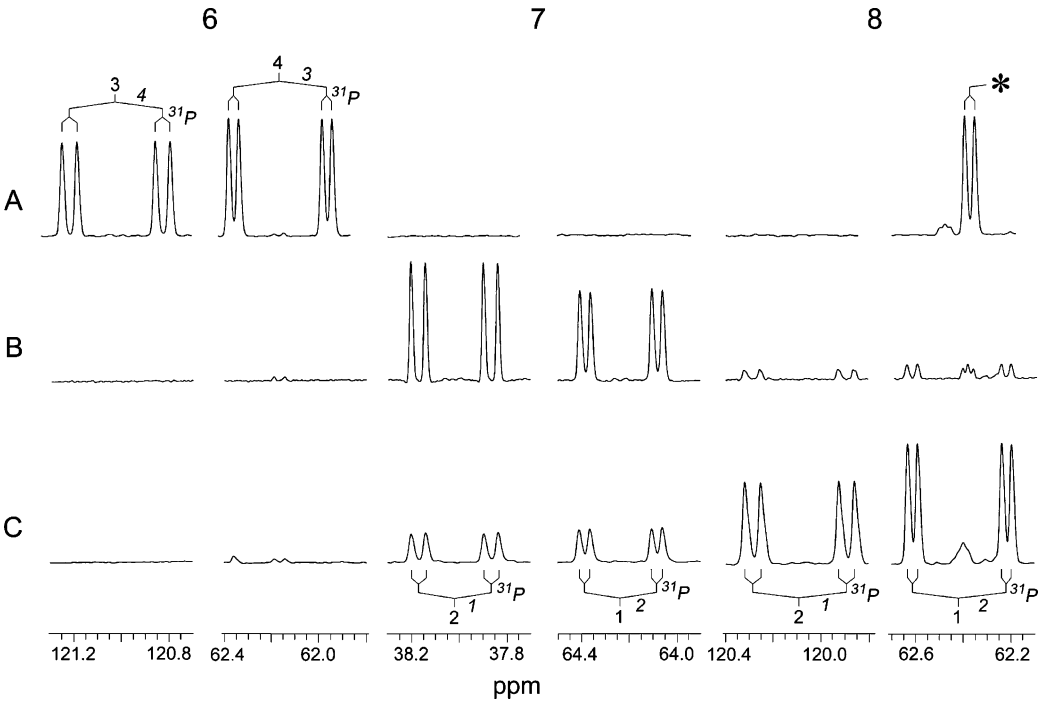

Figure 9. ^{13}C NMR signals detected in a reaction mixture containing [3,4-$^{13}C_2$]-**6** after incubation with IspH protein and subsequent incubation with Idi-1 protein. A, before and B, after incubation with recombinant IspH protein; C, after incubation of sample B with recombinant Idi-1 protein. * indicates the downfield signal component of C-4 of [3,4-$^{13}C_2$]-**6** which resonates at a chemical shift similar to that of C-1 of [1,2-$^{13}C_2$]-**7**.

coupling. The ratio of **7** to **8** as defined by the ratio of the NMR signal integrals was 6.3:1, in line with earlier in vivo and in vitro studies on the formation of **7** and **8** by the catalytic action of IspH protein.[32,34,35] The catalytic rate determined by ^{13}C NMR spectroscopy using [3,4-C_2]-**6** as substrate was 0.66 μmol min^{-1} mg^{-1} in good agreement with assays conducted with radiolabeled **6**. The reaction could be followed to completion; in the assay mixture, the substrate **6** had virtually disappeared after incubation at 37 °C for 1.7 h (cf. Figure 9B). Upon treatment of this reaction mixture with Idi-1 protein, the ratio of **7**/**8** was shifted to the equilibrium value of 1:3.1 (cf. Figure 9C).

Earlier studies had shown that electrons for the IspH-catalyzed reaction can be supplied efficiently by photoreduced deazaflavin.[32,35,36] IspH assays with photoactivated deazaflavin as electron donor were conducted with [3,4-$^{13}C_2$]-**6**; monitoring of the reaction by ^{13}C NMR spectroscopy indicated a catalytic rate of 3.4 μmol min^{-1} mg^{-1} and a partitioning ratio of **7**/**8** of 4.5:1. IspH protein which had been subjected to deazaflavin-sensitized ultraviolet irradiation in the presence of dithiothreitol and in the absence of the substrate **6** showed no catalytic activity when subsequently supplied with a mixture of radiolabeled **6**, flavodoxin, flavodoxin reductase and NADPH.

IspH proteins from a wide variety of organisms show 3 absolutely conserved cysteine residues.[35] To assess the functional roles of the cysteines C12, C96, and C197 in the IspH protein, three serine point mutations were prepared, and the mutant genes were expressed in *E. coli* strains engineered for simultaneous overexpression of the *isc* operon. The enzymatic activity of each of the mutant proteins was below the detection limit and less than 0.0015% as compared to the wild-type protein. The iron content per subunit was 0.6, 0.9, and 1.4 for the C12S, C96S, and C197S mutant proteins, respectively (cf. Table 5).

Table 5. Catalytic Properties of IspH Mutant Proteins

mutation	iron content (mol/mol)	catalytic activity (nmol min^{-1}mg^{-1})	%
none	2.6	700	100
C12S	0.6	<0.01	<0.0015
C92S	0.9	<0.01	<0.0015
C197S	1.4	<0.1	<0.0015

Discussion

The genome of *E. coli* is believed to specify approximately 50 iron−sulfur proteins[48] which are involved in redox reactions but also in various nonredox processes.[49] To become functionally competent, the respective apoproteins require the implementation of iron−sulfur clusters which must be assembled by a complex biochemical machinery involving several proteins. *E. coli* uses at least two different production pipelines for this purpose.[50] The respective genes are clustered in the *isc* and the *suf* operons.[50] Whereas homologues of at least parts of the *isc* operon are found in all organisms, the complete *isc* operon appears to be confined to mitochondria and to α-, β-, and γ-proteobacteria.[51] Orthologous genes of the second iron−sulfur assembly cluster, the *suf* operon, are also widespread in bacteria, Archaea and plastids.[50] Due to the redundancy of the iron−sulfur pipelines, the deletion of either operon is nonlethal in *E. coli*, whereas the orthologs of the *isc* genes are essential in yeast where only one assembly pipeline exists for iron−sulfur clusters.

As shown repeatedly, the massive overexpression of recombinant iron−sulfur proteins can overwhelm the synthetic capacity of iron−sulfur assembly pipeline[52,53] In such cases, the deficit

(47) This finding is in contrast to the data reported in our earlier studies.[35]
(48) Tokumoto, U.; Takahashi, Y. *J. Biochem.* **2001**, *130*, 63−71.
(49) Beinert, H.; Holm, R. H.; Münck, E. *Science* **1997**, *277*, 653−659.
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(51) Mühlenhoff, U. Lill, R. *Biochim. Biophys. Acta* **2000**, *1459*, 370−382.
(52) Nakamura, M.; Saeki, K.; Takahashi, Y. *J. Biochem* **1999**, *126*, 10−18.

Table 6. Biochemical Properties of Recombinant IspH Proteins from Various Sources and under Different Conditions

organism	rec. enzyme	purification	specific activity(μmol min^{-1} mg^{-1}) Fpr/FldA[a]	DAF[b]	DT[c]	iron per subunit (mol/mol)	ϵ_{410} (M^{-1} cm^{-1})	E_{410}/E_{280}	ref
E. coli	maltose binding fusion	aerobically	0.003	0.4	n.d	0.3[d]	n.d.	0.12	32
E. coli	histidine-tagged	aerobically	0.002	0.011	n.d.	0.8[e]	n.d.	0.14	36
E. coli	histidine-tagged	aerobically, reconstituted with Na_2S, $FeCl_3$ chloride and dithiothreitol	0.044	0.030	n.d.	5.0[e]	18,750[f]	0.53	36
E. coli	histidine-tagged	anaerobically from an *isc* operon hyperexpressing strain	0.7	3.4	n.d.	2.6[g]	11,800[h]	0.38	this study
A. aeolicus	histidine-tagged	anaerobically	n.d.	n.d.	6.6	n.d.	n.d.	n.d.	33

[a] Assayed with flavodoxin (FldA) and flavodoxin reductase (Fpr) as electron donors. [b] Assayed with photoreduced deazaflavin (DAF) as electron donor. [c] Assayed with dithionite (DT) as electron donor at 60 °C. [d] Calculated for a molecular subunit mass of 77 715 Da. [e] Calculated for a molecular subunit mass of 36 591 Da. [f] Referenced on a dimeric protein structure. [g] Calculated for a molecular subunit mass of 36 173 Da. [h] Referenced on a monomeric protein structure.

can be compensated by the overexpression of the relevant iron−sulfur assembly Isc proteins.

The expression of IspH apoprotein in *E. coli* is per se not dependent on a sufficient supply of iron−sulfur clusters. In fact, the expression of IspH apoprotein is somewhat reduced by the coexpression of *isc* proteins. Nevertheless, the specific activity and the iron content of the protein are both significantly increased by the coexpression of the *isc* cluster. This is well in line with the hypothesis that the availability of iron−sulfur clusters is the limiting factor for the formation of fully functional IspH protein in IspH hyperexpression strains of *E. coli*.

The specific activity of the protein from the *isc/ispH* hyperexpression strain was in the range of 0.5−0.7 μmol min^{-1} mg^{-1} as established by three different assay methods. The specific advantages of each of these assays deserve a brief discussion. (i) The main advantage of the radiochemical assay, especially when performed with substrate of very high specific radioactivity, is its high sensitivity; thus, this assay could be used to show that the replacement of any of the three absolutely conserved cysteine residues in IspH protein results in an activity loss of at least 70 000-fold. (ii) The photometric assay measuring the consumption of the cosubstrate, NADPH, has the advantage of simplicity, and this assay method appears suitable for the screening of IspH inhibitors as potential anti-infective agents from chemical libraries by high throughput screening methods. (iii) The main advantage of the NMR method is its exquisite selectivity. Thus, **7** and **8** can be assessed individually, and the ratio of their formation can be determined with high precision. The inherently low sensitivity of ^{13}C NMR spectroscopy can be compensated to some extent by selective ^{13}C-labeling of the substrate. The labeling of two adjacent carbon atoms of the substrate used in the present study adds additional selectivity, because each carbon atom now appears as a doublet due to $^{13}C^{13}C$ coupling; the slight sensitivity loss caused by splitting of the signals can be tolerated.

Properties reported in the literature for different preparations of the IspH protein together with the data from the present work are summarized in Table 6. The catalytic activity of protein purified under aerobic conditions from an IspH hyperexpression strain of *E. coli* (without hyperexpression of *isc* genes) reported in our earlier study,[32] when assayed with auxiliary proteins under quasi-natural conditions, was only about 0.5% as compared to the protein preparations reported in this paper. In comparison with the activity observed in the present study, the activity of IspH protein isolated under aerobic conditions by Rohmer and co-workers[36] was 0.3% before and 6.3% after in vitro reconstitution with ferric chloride, sodium sulfide and dithiothreitol. A recombinant IspH protein of the thermophile *A. aeolicus* was reported to have a catalytic activity of 6.6 μmol min^{-1} mg^{-1} with dithionite as an artificial reducing agent at 60 °C.[33] However, the homologous protein studied here did not show any activity under the conditions described in ref 33.

In contrast to previous studies,[36] the enzyme described in this report had been naturally loaded with iron and was not subjected to any artifactual procedures before biochemical and spectroscopic characterization. The presence of an iron−sulfur center in the as-isolated protein was detected by UV spectroscopy (see above) and was confirmed by EPR spectroscopy. Both the line shape and the spectral position determined by the *g*-matrix of the observed EPR signal (Figure 5, traces A and B) are characteristic for a $[3Fe\text{-}4S]^+$ center bound to a protein environment. This becomes evident by comparison of the *g*- and line width parameters from a simulation with those from other protein-bound $[3Fe\text{-}4S]^+$ centers, e.g., in anaerobic ribonucleotide reductase,[54] in family-4 uracil-DNA glycosylase,[55] and in the tRNA-modifying enzyme MiaB.[56] For a sample of the IspH protein isolated under aerobic conditions, an EPR signal of similar line shape has previously been reported by Rohmer and co-workers and assigned to a $[3Fe\text{-}4S]^+$ center, which they interpreted as an oxidative degradation product of the native cluster.[36] Upon reconstitution of the protein with ferric chloride, sodium sulfide and dithiothreitol under anaerobic conditions and subsequent reduction with dithionite, a broad EPR signal was observed as the only paramagnetic species and assigned to a $[4Fe\text{-}4S]^+$ center on the basis of its characteristic *g*-principal values. From their activity measurements, Rohmer and co-workers concluded that this $[4Fe\text{-}4S]^{2+/+}$ cluster is the prosthetic group in IspH. In the experiments with the as-isolated IspH protein described in this work the nature of the competent entity

(53) Schwarz, C. J.; Djaman, O.; Imlay, J. A.; Kiley, P. J. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 9009−9014.
(54) Ollagnier, S.; Mulliez, E.; Schmidt, P. P.; Eliasson, R.; Gaillard, J.; Deronzier, C.; Bergman, T.; Gräslund, A.; Reichard, P.; Fontecave, M. *J. Biol. Chem.* **1997**, *272*, 24216−24223.
(55) Hinks, J. A.; Evans, M. C. W.; de Miguel, Y.; Sartori, A. A.; Jiricny, J.; Pearl, L. H. *J. Biol. Chem.* **2002**, *277*, 16936−16940.
(56) Pierrel, F.; Björk, G. R.; Fontecave, M.; Atta, M. *J. Biol. Chem.* **2002**, *277*, 13367−13370.

generated by reduction of the original $[3Fe\text{-}4S]^{+}$ cluster remains elusive.

The IspH protein does not display the characteristic ferredoxin signature of many other [4Fe-4S] cluster binding proteins, but we have now verified by the use of specific serine mutants that the substitution of any of the three strictly conserved cysteine residues of the enzyme results in a dimished capacity to bind iron ions and in the loss of enzymatic activity. The strict anaerobic conditions maintained during the preparation of the protein rule out that in this case the detected $[3Fe\text{-}4S]^{+}$ is a product of oxidative degradation. By analogy with the known cases of aconitase[57] and the pyruvate-lyase activating enzyme,[58] formation of this cluster could be explained by the loss of a labile iron center not coordinated with cysteine in a preformed [4Fe-4S] precursor. In the specific case of the pyruvate-lyase activating enzyme it has been demonstrated by Mössbauer spectroscopy that the enzyme isolated under anaerobic conditions contains, next to predominant amounts of a $[3Fe\text{-}4S]^{+}$ cluster, lower amounts of $[4Fe\text{-}4S]^{2+}$, $[2Fe\text{-}2S]^{2+}$, and linear $[3Fe\text{-}4S]^{+}$ clusters; moreover, it could be shown that upon treatment with dithionite the mixture undergoes a remarkable conversion into the cuboidal [4Fe-4S] form, mainly in the diamagnetic $[4Fe\text{-}4S]^{2+}$ state, whereas exposure of the same mixture to photoreduced deazaflavin resulted mainly in the formation of the paramagnetic $[4Fe\text{-}4S]^{+}$ state, which was claimed to represent the real catalytic reducing agent.[58] This important observation illustrates the complexity of the problem and offers a possibility for reconciling our results with the ones previously reported for the artificially reconstructed form of the enzyme.[36] The striking differences (cf. Table 6) in the specific activities of both preparations remain somewhat puzzling.

The ratio of **7**/**8** formed by IspH protein in vivo[34] and under in vitro assay conditions with auxiliary redox shuttle proteins is 6.3:1, whereas the ratio at equilibrium is 1:3.1 (as determined by NMR analysis of IspH product mixtures before and after treatment with IPP isomerase). It follows that the ratio of the two products formed by IspH is kinetically controlled. In line with that, the partitioning factor for the formation of **7** and **8** under the catalytic action of IspH protein appears to be invariant over the entire reaction period within the accuracy limits of NMR observation.

In an artifactual experimental setup, the IspH-catalyzed reaction can be driven by photoreduced deazaflavin as electron source. As in our previous experiments,[32,35] the specific activity was higher in the artifactual system as compared to the more physiological setup using electron shuttle proteins such as flavodoxin, ferredoxin, and the cognate reductases. However, we can rule out the possibility that the ultraviolet irradiation in the presence of deazaflavin is conducive to activation of the protein per se; in fact, deazaflavin-sensitized ultraviolet irradiation in the absence of substrate destroyed the enzyme's ability to catalyze the formation of **7** and **8** with a mixture of **6**, flavodoxin, flavodoxin reductase and NADPH as cofactors.

Table 7. Partitioning Factors for IPP and DMAPP Reported in the Literature and in the Present Study

partitioning factor	ref
4−5:1	33
5:1	34
∼6:1	35
4−6:1	36
4.5[a] and 6.3 [b]:1	this study

[a] Determined in assays with reduced deazaflavin as electron donor. [b] Determined in assays with flavodoxin and flavodoxin reductase as electron donors.

The increased sensitivity of the present methods enabled a verification of the partitioning factor which governs the parallel formation of **7** and **8**. Values from the literature and from the present study are summarized in Table 7. Small, but significant differences are systematically observed between experiments carried out under quasi-natural experimental conditions with auxiliary redox shuttle proteins and under artifactual conditions with photoreduced deazaflavin, respectively dithionite. Such differences are taken to indicate that the transition states for the conversion of **6** into products **7** and **8** are not strictly identical in the two different experimental setups.

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Supporting Information Available: Calculated molecular masses and isoelectric points of proteins from the *isc* operon. Inactivation of recombinant IspH protein by divalent metal ions. This material is available free of charge via the Internet at http://pubs.acs.org.

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