Synthesis and characterization of de novo designed peptides modelling the binding sites of [4Fe–4S] clusters in photosystem I

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** Abbreviations: Chl a, Chlorophyll a; CW, Continuous Wave; DCPIP, 2,6-dichlorophenolindophenol; DIFPEA, N,N-dioctanoylphthalamine; j-DM, N-dodecyl-β-D-maltoside; ENDOR, Electron Nuclear D(d)e Double Resonance; EPR, Electron Paramagnetic Resonance; ESEEM, Electron Spin Echo Envelope Modulation; HYSCORE, HYperSpectral ResoRance; HPLC, High Performance Liquid Chromatography; NADP, nicotinamide adenine dinucleotide phosphate; NMP, N-methylpyrrolidone; MALDI-TOF-MS, Matrix Assisted Laser DesorptionIonisation – Time-of-Flight – Mass Spectrometry; P700-FX, core; PsA, Photosystem I.

1. Introduction

Iron–sulfur clusters are ubiquitous in biology and play many different roles in the living cell (reviewed in [1–5]). They act as catalysts, sensors and transcription regulators and play a signalling role during DNA repair. By far most common is their participation as catalysts, sensors and transcription regulators and play a signalling role during DNA repair.

Owing to their biological significance, iron–sulfur clusters have been extensively modelled in the past. A large body of literature exists on the investigation of chemically synthesized model compounds, mimicking iron–sulfur clusters of different nuclearity, in organic solvents (for a recent review see [7]). While providing valuable insight into the chemistry of the iron–sulfur clusters, the majority of these models cannot account for the interactions of the iron–sulfur cluster with its protein binding site, which includes interaction with non-ligating amino acids or with surrounding water molecules. Both types of interactions are believed to strongly influence the physiologically relevant properties of iron–sulfur clusters, e.g., redox potential and catalytic activity.

In the past two decades several attempts were made to prepare models containing peptide or protein ligated iron–sulfur clusters in aqueous buffers [8–17]. Initially, the entire polypeptide sequence of ferredoxin from Clostridium pasteurianum [14,15], and a truncated polypeptide sequence from Desulfovibrio gigas ferredoxin II [16] were synthesized in vitro. More recently two main approaches for modelling binding sites were explored: the first relies on designing soluble protein, as for ferredoxins, or as part of an electron transfer chain in a large protein or protein complex, as in hydrogenase or photosystem I. A wide variety of different iron–sulfur clusters exists in biology. The inventory of unique protein folds for different iron–sulfur proteins was recently compiled by Meyer [6].

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synthetic peptides containing the conserved iron–sulfur binding site [8–11]; the second relies on the introduction of the iron–sulfur cluster binding site into a naturally occurring or synthetic protein that was previously incapable of binding an iron–sulfur cluster [8,12,13,17]. It should be noted, however, that up to date only peptide-ligated models of [4Fe–4S] clusters were reported in the literature.

Incorporation of low-potential [4Fe–4S]$^{2+}/^{1+}$ clusters into peptides of different sizes was investigated with the aim of determining the minimal requirements for successful binding [8–10]. Mulholland et al. investigated the influence of the amino acid composition of model peptides on the binding efficiency of iron–sulfur clusters [9,10]. In this study several model peptides with lengths between 4 and 16 amino acids were investigated. It was found that aside from the presence of a consensus iron–sulfur binding motif, containing at least three cysteines, which are appropriately spaced (CxxCxxC), the choice of non-liganding amino acids plays a decisive role in the efficiency of [4Fe–4S] cluster incorporation. By analysis of the amino acid sequence of 510 naturally occurring ferredoxins the prevalence of specific non-ligand amino acids in certain sequence positions within the consensus binding motif was established [10]. It was shown that β-branched amino acids like Ile or Val are dominant in the second position and Gly in the third and the fifth position. There seems to be less restriction for the choice of the sixth non-ligand amino acid in the consensus iron–sulfur cluster binding motif. While the apolar Ala is prevalent in this position (18%), a positively charged Arg is the second most prominent (12%) and Gln is also relatively common (8%).

Despite the variation of the amino acid composition, all previously studied maquettes containing a ferredoxin binding site show similar biophysical and biochemical properties, namely identical EPR spectra and a redox potential of about −0.350 V [8–10], or even higher, up to −0.289 V [11].

We consider that the next logical step is to model [4Fe–4S] cluster(s) which function within a large protein complex and whose biological role is well-known. Therefore, our attention turned to photosystem I (PS I), where the crystal structure of the overall complex is known [18], and the function of the [4Fe–4S] clusters in the electron transfer chain have been studied in detail for over 30 years and is well-established (see recent reviews collected in [19]).

PS I is a membrane-bound, multi-cofactor, energy-transforming protein complex, that is an indispensable part of the photosynthetic electron transfer chain in plants and cyanobacteria. PS I is a Type I reaction center, where the terminal electron acceptor is a [4Fe–4S] cluster. Three low-potential [4Fe–4S] clusters are bound on the reducing (stromal) side of PS I, usually referred as F_A, F_B and F_C (reviewed in [20]).

The [4Fe–4S] cluster F_C is an unusual case of an interpolyptide iron–sulfur cluster, with two cysteine ligands provided by the PsaA subunit and two by the PsaB subunit of PS I. The binding site of F_C is identical on both subunits. Interestingly, F_C has one of the lowest midpoint reduction potentials known for a [4Fe–4S] cluster, values ranging from −0.730 V [21] to −0.705 V [22] (all potentials versus SHE). Scott et al. incorporated a binding motif of PS I [4Fe–4S] cluster F_C into the 4-α-helix bundle designed by the group of DeGrado [13]. This is the first, and so far the only model of both an iron–sulfur cluster involved in photosynthesis and of a interpolyptide iron–sulfur cluster. It has an EPR spectrum nearly identical to maquettes containing the ferredoxin binding site, which is quite different from the EPR spectrum of the interpolyptide [4Fe–4S] cluster F_C in PS I. This model, however, exhibits the lowest reduction potential found for a [4Fe–4S] cluster bound to a peptide maquette up to now (−0.422 V).

The iron–sulfur clusters, F_A and F_B, follow F_C in the electron transfer chain of PS I. They are bound to the PsaC subunit of PS I, which is located on the stromal side of the thylakoid membrane (Fig. 1A, for a recent review on PsaC structure and its binding to PS I see [23]). It was shown spectroscopically that F_B is the terminal electron acceptor in PS I [24–28] (reviewed in [20]). The midpoint reduction potentials of F_A and F_B in fully assembled PS I were measured at cryogenic

![Fig. 1.](image-url) (A) Three-dimensional structure of PsaC subunit taken from the X-ray structure of PS I at 2.5 Å resolution (PDB entry 1B0O) [18]. Detail of the structural model of the PS I monomer showing the backbone of the PsaC subunit and the [4Fe–4S] clusters F_A and F_B bound by it. The iron–sulfur clusters are shown as cubes, in which the yellow corners indicate position of sulfur atoms and light-brown corners the position of iron atoms. (B) Amino acid sequence of the designed peptides F_A and F_B (1) compared to the amino acid sequence of the PsaC subunit of PS I from Synechococcus sp. PCC 7002 (2) and the consensus low-potential [4Fe–4S] cluster binding motif (3). Numbering refers to the PsaC sequence. The Lys 51 and Arg 52 are crucial for binding of PsaC within PS I via the formation of salt bridges with amino acids on the PsaA and PsaB subunits [23,36]. They are shown as “stick” models in (A) and identified by the light blue box in (B).
temperature by redox titrations using EPR detection and were found to be $-0.540$ V and $-0.590$ V, respectively [29,30]. However, by titration of isolated PS I complexes using optical detection of the charge recombination between the iron–sulfur clusters and P700$^+$ at room temperature, potentials of $-0.465$ V and $-0.440$ V were found for FA and FB, respectively [31]. The latter potentials are closer to typical midpoint redox potentials found for [4Fe–4S] clusters in bacterial dicluster ferredoxins. It should be noted that in unbound PsA FA and FB have a very similar midpoint reduction potential (ca. $-0.460$ V [J.H. Golbeck, personal communication]), which is different from the one in PS I-bound PsaC. It is impossible to obtain an independent reduction potential for either of the clusters in the unbound PsaC, most likely due to the fast exchange of an electron between the FA and FB clusters [32]. Note that PsaC experiences significant changes in its three-dimensional structure upon binding to the PS I core, especially in the presence of the PsaD subunit. These changes result in different EPR spectra of PS I-bound FA and FB and their altered reduction potentials [23,33–36]. Thus the spectroscopic and biochemical properties of the [4Fe–4S] clusters FA and FB are well-studied. The protein environment of both iron–sulfur clusters is also structurally well-characterized [18,35]. This makes FA and FB good studied. The protein environment of both iron

2. Materials and methods

2.1. Synthesis of apo-FA and apo-FB peptides

Peptide synthesis was performed on an Advanced Chemtec 348Ω synthesizer. All chemicals were purchased from Iris Biotech and were used without further purification. PAL-PEG-PS resin was purchased from Applied Biosystems. The following side chain protection scheme was applied: tert-butyl: Thr, Asp, Glu, Tyr; trityl: Gln, Gln. Puriﬁcation was achieved by ultraﬁltration in an Amicon stirred cell (Millipore Corp.) furnished with a 1 kDa cut-off membrane (YM-1). Excess of unbound low molecular weight compounds was removed by passing the sample twice through a pre-packed gel-ﬁltration column with Sephadex G-25 as solid phase (PD 10, GE Healthcare), where the dark brown fraction was collected. The sample was further concentrated by ultraﬁltration as described above.

2.2. Preparation of the C50G C33S variant of PsaC

The apo C50G C33S variant of the PsaC subunit of PS I was overproduced in Escherichia coli and puriﬁed as described previously [34].

2.3. Iron–sulfur cluster insertion

The [4Fe–4S] clusters were inserted into the apo-FA and FB peptides and apo C50G C33S PsaC by a previously described procedures [35,37–39], which is an adaptation of the original protocol of Lovenberg et al. [40]. Briefly, to 50 mM Tris/HCl, pH 8.3, 0.8% vol/vol 2-mercaptoethanol buffer a solution of 1 mg/ml apo peptide (or 5 mg/ml for apo PsaC) was added to a ﬁnal peptide/protein concentration of 10 μM. This was followed by the dropwise addition of a 60 mM iron(III)chloride and a, freshly prepared, 60 mM sodium sulfide solutions to the ﬁnal concentrations of 180 μM each. All additions were done in 20 minute intervals. All solutions and the Tris/HCl buffer were degassed and purged with argon prior to use. The reconstitution reaction was allowed to incubate overnight at 279 K. Then it was transferred to a Coy anaerobic chamber (COY Inc., Grass Lake, MI, USA) and all further manipulations were performed anaerobically. The iron–sulfur insertion reaction mixture was concentrated by ultraﬁltration in an Amicon stirred cell (Millipore Corp.) furnished with a 1 kDa cut-off membrane (YM-1). Excess of unbound low molecular weight compounds was removed by passing the sample twice through a pre-packed gel-ﬁltration column with Sephadex G-25 as solid phase (PD 10, GE Healthcare), where the dark brown fraction was collected. The sample was further concentrated by ultraﬁltration as described above.

2.4. kPreparation of reduced samples of FA and FB peptides and the C13G C33S and C50G C33S variants of PsaC

Prior to reduction of iron–sulfur clusters, the pH of the samples was adjusted to 10.0 by addition of 1 M glycine buffer to a ﬁnal concentration of 300 mM. Then a freshly prepared stock solution of 300 mM sodium dithionite was added to a ﬁnal concentration of 30 mM. All manipulations were done inside the anaerobic chamber.

2.5. Preparation of $^{57}$Fe enriched samples

$^{57}$FeCl$_3$ was prepared as follows: a known amount of solid $^{57}$Fe was dissolved in concentrated HCl in electric contact to a sheet of platinum metal. After the reaction was complete (24–48 h), the solution was dried under vacuum. The yellow to red solid was dissolved in buffer inside the anaerobic chamber and the concentration of $^{57}$FeCl$_3$ was calculated from the initial amount of solid $^{57}$Fe. $^{57}$FeCl$_3$ was used, in place of unlabeled iron (III) chloride, in iron–sulfur cluster insertion into the apo-FA and FB peptides and the apo C50G C33S PsaC variant using procedures described above.

2.6. Optical absorption spectroscopy

UV-Vis spectra were recorded on an ATI Unicam spectrometer model UVS-200, which was controlled by Thermo Scientiﬁc software version 1.25. For all measurements gas-tight quartz cells with 1 cm path length were used. To determine the peptide concentration a calculated extinction coefﬁcient of 5500 M$^{-1}$ cm$^{-1}$ for FA and 6990 M$^{-1}$ cm$^{-1}$ for FB was used [41]. The yield of iron–sulfur cluster incorporation was calculated using an extinction coefﬁcient of ε$_{430}$ = 16,000 M$^{-1}$ cm$^{-1}$ [42].

2.7. Continuous wave (CW) electron paramagnetic resonance (EPR) spectroscopy

CW EPR samples were measured on a Bruker E 500 spectrometer operating at X-band frequency. The temperature was controlled by an
Oxford ESR continuous flow cryostat model 910 combined with an Oxford ITC 503 intelligent temperature controller. The magnetic field was calibrated using a Bruker NMR Gaussmeter ER 035 M. Typical conditions for EPR measurements were: sample temperature 15 K, microwave frequency ca. 9.4 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 10 G and time constant 40 ms, 5 scans. Simulations of holo Fα and holo Fβ EPR spectra were performed using the EasySpin software [43,44], using the combination of Lorentzian and Gaussian linewidth broadening (pseudo-Voigt function) in order to represent natural linewidth and unresolved hyperfine structure of the EPR lines. In addition for better representation of the lineshapes anisotropic g strain was added.

2.8. Mössbauer spectroscopy

Mössbauer spectra were recorded on a spectrometer equipped with a Varian cryostat made by Oxford Instruments, and operating in the usual constant acceleration mode. The minimal experimental linewidth was 0.24 mm/s. The source was $^{57}$Co in a 6 µm rhodium-matrix. Isomer shifts are referenced to α-iron at 300 K. All measured samples were enriched with $^{57}$Fe.

2.9. Pulse EPR, ENDOR

2.9.1. Three pulse ESEEM measurements

Measurements were performed on the X-band Bruker Elexsys 580 spectrometer equipped with a dielectric cylindrical resonator. The temperature was controlled by an Oxford ITC liquid helium flow system. In all experiments the temperature was 10 K and the microwave frequency ca. 9.7 GHz. The length of all π/2 pulses was 16 ns. The delay time τ was 80 ns. The echo was integrated with a time window of 4 ns. The shot repetition rate was 1.5 ms. Prior to Fourier transformation the data were corrected using a polynomial base line. The cross-term averaging algorithm [45] was used to improve the phase stability of the Fourier transformed data.

2.9.2. Q-band ENDOR measurements

Measurements were performed using Q-band Bruker Elexsys 580 equipped with home-built cylindrical resonator [46,47]. The sample temperature was 6 K. A Davies ENDOR sequence with a 200 ns inversion pulse and a π/2−τ−π echo detection sequence (36 ns and 72 ns pulses, $\tau = 420$ ns) were used. A 15 µs radiofrequency pulse was applied. The repetition time was 5 ms.

2.10. Potentiometric titration of model peptides with bound iron-sulfur clusters

All potentiometric titrations were performed inside an anaerobic chamber. As a reference electrode, a home-built 1 M Ag/AgCl electrode (1 M KCl) was used, the working electrode was made of Pt wire. A calibrated high-impedance voltmeter was used to connect reference and working electrode (Radiometer, Copenhagen, Denmark). To rule out an error of the measured redox potential due to a hindered electrochemical contact between the solution and the electrode surface, the potentiometric titration of each peptide was repeated in the presence of mediators. However, the presence of the redox mediators such as indigo-tetrasulfonate ($-0.046$ V), phenosafranine ($-0.252$ V) and methyl viologen ($-0.448$ V) had no effect on the determined potential or on the overall titration.

2.10.1. UV-visible detection

The peptide solution was titrated with a solution of sodium dithionite in 1 M glycine buffer at pH 10. To the continuously stirred peptide solution an aliquot of 60 mM sodium dithionite was added and after a constant potential was reached, an aliquot was used to monitor the UV/Vis absorption. The titration was continued until an excess of sodium dithionite was visible at 315 nm in the UV/Vis spectra. This corresponds to a complete reduction of the sample. During the titration, aliquots were taken and several EPR samples were prepared and immediately frozen in liquid nitrogen to get an independent evidence of the reduction progress.

2.10.2. EPR detection

Prior to reductive titration, the reconstituted samples were adjusted to pH 10.0 using a stock solution of 1 M glycine buffer (pH 10.0). The final concentration of glycine was 300 mM. Sodium dithionite (300 mM Na$_2$S$_2$O$_4$ in 1 M glycine (pH 10.0)) was added in 1 µl aliquots to a continuously stirred solution of reconstituted peptide ($-1.5$ mM). The potential values were recorded after a steady potential reading was achieved. After each measurement, an aliquot of the peptide sample was removed and frozen in liquid nitrogen for EPR measurements.

The relative fraction of reduced [4Fe–4S] clusters was calculated based on the decrease of UV/Vis absorption at 410 nm or from the increase in amplitude of the EPR signal at g$_2$. Midpoint potentials were calculated by fitting the fraction of reduced [4Fe–4S] clusters versus the ambient potential of the solution to the Nernst equation for a one-electron transfer per oxidation/reduction process using a nonlinear Marquardt regression algorithm.

2.11. Time-resolved optical spectroscopy at 820 nm

The samples for kinetic measurements were prepared anaerobically in 50 mM Tris buffer (pH 8.3) containing 4 mM sodium ascorbate and 0.04% n-dodecyl-β-D-maltoside [@-DM] at Chl a concentrations of 70–100 µg/ml. Flash-induced absorbance changes were measured in the μs-to-s time range at 820 nm with a laboratory-built double-beam spectrometer as described previously [48]. The actinic flash was provided by a frequency-doubled, Nd-YAG laser (λ = 532 nm, 7 ns pulse duration, flash energy of ~2–3 mJ/cm², Quanta-Ray DCR-11, Spectra Physics, CA). Typically, 12 to 16 transients were recorded and averaged. Multieponential fits of the kinetic data were performed using the PLUK software [49]. The best solution of the fitting was chosen based on the analysis of the residuals of the fits, the probability and correlation matrix for all fitted parameters.

2.12. Preparation of photosystem I, P700-Fα cores

The preparation of thylakoid membranes, the isolation of trimeric PS I complexes from Synechococcus sp. PCC 7002 using [@-DM] and the purification were done according to previously published procedures [50]. PS I complexes without Fα and Fβ clusters (named P700-Fα cores) were isolated from PS I as described before [48]. Removal of the stromal subunits Psαd, Psαe, and Psαc with the terminal iron–sulfur clusters Fα and Fβ, was followed by monitoring the kinetics of re-reduction of P700$^+$ at 820 nm. Independently, the removal of Fα and Fβ clusters was verified by EPR spectroscopy (15 K, microwave frequency 9.436 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 10 G). The sample was prepared by freezing of P700-Fα core in the presence of 10 mM of sodium ascorbate and 50 µM DCPPIP under continuous illumination with a 150 W halogen lamp.

2.13. Rebinding of holo Fα and Fβ peptides to P700-Fα core

For reconstruction of the P700-Fα core with the holo Fα or holo Fβ peptide, P700-Fα core was incubated overnight in the dark on ice with a 10 times molar excess of the respective holo peptide. The non-bound peptide was then removed by repeated dilution-concentration on a Nanosep-100 concentrator with a 100 kDa molecular weight cut-off membrane (Pall Corp.). All manipulations were done inside an anaerobic chamber.
3. Results

3.1. Design of model peptides

The Psac subunit of PS I is presumed to have evolved from bacterial ferredoxins which bind two [4Fe–4S] clusters (dicluster ferredoxins). However, the sequence similarity to them is restricted to having two [4Fe–4S] cluster binding motifs C(1)xxC(II)xxC(III)xxC(IV)P and C(1′)xxC(II′)xxC(III′)xxC(IV′)P. Cysteines I, II, III and IV′ ligate the first iron–sulfur cluster (F₈) and cysteines 1′, II′, III′ and IV′ ligate the second iron–sulfur cluster (F₉) (reviewed in [23]) (Fig. 1). In our design approach these two ligation schemes are separated into two distinct peptides. The obvious point to dissociate the two binding sites is between cysteine IV and cysteine 1′. In a second step the fourth ligand for each binding site needs to be relocated so that it lies in close vicinity to the first three cysteines. This can be achieved by introducing a short loop consisting of the residues KPE, where Pro is supposed to initiate the loop and the residues lysine and glutamate form a salt bridge to stabilize the preformed turn. This approach has been successfully used before [9]. Additionally, three preceding residues in front of the first cysteine have been introduced in order to provide additional shielding of the cluster and to avoid side reactions of the N-terminal cysteine residues during peptide synthesis. The peptide modelling the binding site of the F₈ cluster of Psac is named F₈ peptide and the peptide modelling the binding site of the F₉ cluster of Psac is named F₉ peptide. Except for the designed loop region, the native amino acid sequence of Psac from Synechococcus sp. PCC 7902 has been used, i.e. Tyr 7 to Cys 16 for the sequence YDTICTQCKKPECPW (apo-F₈ peptide); Thr 44 to Cys 53 for the sequence TEDCVGKRCKKPECPW (apo-F₉ peptide) (Fig. 1B).

3.2. Peptide synthesis and iron–sulfur cluster insertion

The apo-F₈ and apo-F₉ were synthesized by the standard Fmoc-routine with an overall yield of 17% each. Neither a prolonged reaction time during amino acid coupling nor a change of coupling reagent had any significant effect on the yield. The relatively low yield of the synthesis may be due to the high abundance of Cys residues (four Cys out of 16-amino acids). Cysteine is known to undergo several reactions during peptide synthesis [51]. The purity of the synthesized apo-peptides was controlled by analytical HPLC (>99%) and MALDITOF-MS (data not shown). The determined mass of each peptide corresponds to the calculated value within error: F₈ peptide — found 1850.5 (calculated 1852.8), and F₉ peptide — found 1845.6 (calculated 1845.7).

The extinction coefficient of both apo-peptides was estimated using the equation derived by Pace et al. [41]:

\[ \varepsilon_{280} (M^{-1} \text{ cm}^{-1}) = 5500(\text{Trp}) + 1490(\text{Tyr}) \]  

(1)

Here (Trp) and (Tyr) represent the number of the respective amino acid in the peptide sequence. In this manner, extinction coefficients of 5500 M⁻¹ cm⁻¹ and 6990 M⁻¹ cm⁻¹ were calculated for apo-F₈ and apo-F₉ peptides, respectively.

Iron–sulfur clusters were inserted into the apo-F₈ and F₉ peptides as described in the Materials and methods section. The peptide is a chelate ligand, containing four cysteine residues, and it is expected to displace all the 2-mercaptoethanol molecules that initially ligate the [4Fe–4S] cluster formed in the reconstitution mixture. This was reported before for reconstitution of the Psac subunit of PS I [35,37,38] and for insertion of iron–sulfur clusters into model peptides [8–10,13].

The reconstituted holo F₈ and holo F₉ peptides show a broad absorption in the visible range of the optical absorption spectrum with a maximum around 410 nm (Fig. 2). This is typical for oxidized iron–sulfur clusters and is ascribed to a sulfur to iron charge-transfer band. The extinction coefficient for a [4Fe–4S] cluster is about 16,000 M⁻¹ cm⁻¹ at the absorption maximum [42]. The ratio of the concentrations calculated using the absorbances at 280 and 410 nm allows a calculation of the efficiency of iron–sulfur cluster reconstitution, which was found to be 24% for F₈ and 12% for F₉.

The absorption at 410 nm is reduced significantly upon reduction of the sample by sodium dithionite, which is characteristic for iron–sulfur proteins. In summary, the UV/Vis spectra of the holo F₈ and holo F₉ peptides in the oxidized and reduced states (Fig. 2) are typical for protein/peptides binding iron–sulfur clusters.

3.3. Investigation of iron–sulfur clusters bound to model peptides by CW EPR spectroscopy

We used CW EPR spectroscopy at X-band to probe the identity of the iron–sulfur–clusters bound to the holo F₈ and holo F₉ peptides. A strong EPR spectrum was detected for the reduced holo F₈ and holo F₉ peptides (Fig. 3). In both cases it can only be simulated by assuming a rhombic g-tensor. The values, determined by simulation, are 2.04 (g₁), 1.93 (g₂), and 1.89 (g₃) for holo F₈; and 2.05 (g₁), 1.93 (g₂), and 1.90 (g₃) for holo F₉. EPR signals of iron–sulfur clusters bound to the holo F₈ and holo F₉ peptides cannot be observed above a temperature of 40 K due to excessive line broadening by fast spin relaxation [52]. The microwave power dependences of the EPR spectra observed for both the holo F₈ and holo F₉ peptides are...
characteristic for the \([4Fe-4S]\) cluster (not shown). The EPR signals of the iron–sulfur clusters can be maximized by variations of the microwave power and temperature. Highest signal intensities are obtained at 18 K and 200 mW for holo FA and at 6 K and 200 mW for holo FB. Thus EPR signals of both holo peptides could not be saturated under our experimental conditions using up to 200 mW of microwave power.

Similar EPR spectra were observed earlier for \([4Fe-4S]\) clusters in single cluster ferredoxins, for model peptides \([8-11]\) and also for PsAC (C13G C33S and CS0G C33S mutants), where one of the iron–sulfur clusters is not detectable in the \(g = 2\) region of the EPR spectrum \([53-55]\).

EPR signals characteristic for high-spin \((S \geq 3/2)\) \([4Fe-4S]\) clusters were not detected in reduced holo FA and holo FB in contrast to variants of PsAC described below \([53-57]\).

Oxidized \(([4Fe-4S]^2\)) clusters are EPR silent due to strong antiferromagnetic coupling leading to a \(S = 0\) ground state. This is also found here for iron–sulfur clusters bound to holo FA and holo FB (see Fig. 3). Only a very weak signal of a \([3Fe-4S]\) iron–sulfur cluster could be detected in the oxidized samples around \(g \approx 2.01\) (Fig. 3) \((g_x = 1.97, g_y = 2.00, \text{ and } g_z = 2.02)\) \([39,52,58]\). Since this signal has a much smaller linewidth than the reduced \([4Fe-4S]\) cluster, the concentration of \([3Fe-4S]\) clusters in our samples is very small. The formation of trace amounts of \([3Fe-4S]\) clusters is common during in vitro iron–sulfur cluster reconstitution and was reported before \([8,13,53]\).

### 3.4. Investigation of holo FA and holo FB peptides by Mössbauer spectroscopy

Zero-field Mössbauer spectra were recorded from \(^{57}\)Fe-enriched samples of the holo FA and holo FB peptides at 80 K (Fig. 4) in order to identify the type and oxidation state of the iron–sulfur clusters (Fig. 4). The parameters obtained from data fits of the spectra with Lorentzian doublets are summarized in Table 1 for both the oxidized and reduced samples.

The Mössbauer sample of holo FA in the oxidized state exhibits a dominating quadrupole doublet with an isomer shift, \(\delta\), of 0.43 mm/s and an electric quadrupole splitting, \(\Delta E_Q\), of 0.98 mm/s. Apparently the iron sites of the corresponding reconstituted iron–sulfur clusters are basically uniform and not distinguishable in the spectrum. The Mössbauer parameters for the corresponding contribution for oxidized holo FA are \(\delta = 0.46\) mm/s and \(\Delta E_Q = 0.97\) mm/s. The moderate quadrupole splitting and particularly the intermediate values of the isomer shift for both peptides, holo FA and holo FB, are characteristic of tetrahedrally coordinated iron sites with four sulfur ligands and delocalized mixed valences of +2.5 \([3,59,60]\). This delocalized mixed-valence state is a unique feature of oxidized cubane \([4Fe-4S]\) clusters in the \(2^+\) state, which formally contain two Fe(II) and two Fe(III) ions. In contrast, other mixed-valence clusters like \([2Fe-2S]^{1+}\) and \([3Fe-4S]^0\) systems do show distinguished iron sites due to (partial) valence localization.

The spectrum of oxidized holo FA is slightly asymmetric, which we have to assign either to minor differences in the site symmetries or charge densities of the corresponding cluster, or to some heterogeneity of the protein preparation. Note that since EPR lines of reduced holo FA are narrower than of holo FA we find the latter one less likely. But like for holo FB, individual contributions to the spectrum of holo FA can also not be resolved within the experimental line width.

The sample of oxidized holo FA shows a minor subspectrum (6% relative intensity) with isomer shift and quadrupole splitting of 1.3 mm/s and 3.00 mm/s in the sample. The very high isomer shift \(\delta > 1\) mm/s exceeds the range expected for iron–sulfur clusters and indicates iron(II) with hard oxo- or hydroxo-ligands. We, therefore, assign the subspectrum to adventitiously bound iron attached to the peptide. Similar species are also found in the spectra for both peptides in the reduced state.

Upon reduction the isomer shift and the quadrupole splitting of holo FA increases by 0.08 mm/s and 0.05 mm/s, respectively. This trend again is typical of cubane iron–sulfur clusters for the reduction from the \(2^+\) to the \(1^+\) state (for a review see \([59]\)). The values \((\delta = 0.51\) mm/s, and \(\Delta E_Q = 1.03\) mm/s) resemble those of reduced ferredoxins \([3,60]\).

To our surprise the zero-field Mössbauer spectrum of reduced holo FA appears to have a single symmetric doublet with a similar change in the isomer shift, 0.06 mm/s as found for holo FA, but with a reduced quadrupole splitting of 0.70 mm/s. Since we do not have further structural information we might only speculate why in this case the

\[\text{...}
\]
reducing electron contributes an electric-field gradient with the opposite sign than for the other contributions to the efg.

In the C13G C33S variant the second cysteine ligand to the [4Fe–4S] cluster FA (C13) is replaced by an external thiolate, and in the C50G C33S the second cysteine ligand to the [4Fe–4S] cluster FA (C50) is replaced by an external thiolate [53,55]. The C13G C33S and C50G C33S PsaC (reduced) [55] 

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \delta ) [mm/s]</th>
<th>( \Delta E_Q ) [mm/s]</th>
<th>Linewidth [mm/s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>holo FA peptide (reduced)</td>
<td>0.50</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td>C13G C33S PsaC (reduced) [55]</td>
<td>0.44</td>
<td>0.95</td>
<td>0.44</td>
</tr>
<tr>
<td>C50G C33S PsaC (oxidized)</td>
<td>0.44</td>
<td>0.95</td>
<td>0.44</td>
</tr>
<tr>
<td>holo FA peptide (oxidized)</td>
<td>0.50</td>
<td>0.97</td>
<td>0.97</td>
</tr>
</tbody>
</table>

Note that in the C13G variant the second cysteine ligand in the consensus binding site of the [4Fe–4S] cluster FA is replaced by an external thiolate [53,55].

3.5. Investigation of holo FA and holo FB peptides by pulse EPR, ESEEM and ENDOR spectroscopies

3.5.1. X-band ESEEM

Electron spin echo detected EPR spectra at X- and Q-band of both reduced holo FA and holo FB peptides as well as the C13G C33S variant of PsaC were recorded (data not shown). In the low frequency range the ESEEM spectra (0 to 5 MHz), multiple signals attributed to \( ^{14}\text{N} \) nuclei are observed. The ESEEM spectra of holo FA and holo FB peptides were recorded using a single set of parameters using the EasySpin software package [43,44]. The \( ^{14}\text{N} \) hyperfine coupling was assumed to be isotropic. The simulation results are presented in the corresponding figure together with the experimental data (Fig. 5B and C; dashed lines). For holo FA we obtained an effective \( ^{14}\text{N} \) hyperfine coupling of 0.9±0.05 MHz, a quadrupole coupling \((e^2Q/h)\) of 3.0±0.1 MHz, and a quadrupole asymmetry parameter \( \eta = 0.70 \pm 0.02 \). For holo FB the effective \( ^{14}\text{N} \) hyperfine coupling is \( 0.7 \pm 0.07 \) MHz and the quadrupole coupling \( 3.1 \pm 0.1 \) MHz. The position of the ridge confirmed our assignment of the ESEEM lines to an \( ^{14}\text{N} \) nucleus in the (+;+) quadrant below \((-2 \text{ MHz} ; +2 \text{ MHz})\). The HYSCORE spectra of holo FA and holo FB were also recorded (data not shown). In the low frequency area those spectra showed a broad correlation ridge from \((+3 \text{ MHz} ; +4 \text{ MHz})\) to \((-4 \text{ MHz} ; +3 \text{ MHz})\) in the (+;+) quadrant, and an unresolved area in the (−;+) quadrant below \((-2 \text{ MHz} ; +2 \text{ MHz})\).
most probably belong to several nuclei with small quadrupole couplings. Unfortunately, the low resolution of the spectra did not allow a more precise analysis of these signals. The signals, in the \((-;+;+;+\) quadrant, cannot belong to the $^{14}$N nucleus with the 3 MHz quadrupole coupling described above, according to our simulations.

The parameters obtained from the simulations of the holo FA and holo FB ESEEM spectra are very similar to those deduced from ESEEM spectra of several different iron–sulfur proteins [63–68]. No these papers [2Fe–2S]$^+$ and [3Fe–4S]$^+$ clusters in proteins were studied. We are unaware of any previous ESEEM investigations of low-potential [4Fe–4S]$^+$ clusters in proteins or model peptides. Couplings, comparable in value to ours, were also determined from ENDOR spectra of iron–sulfur proteins (see for example [69,70]). Independently, similar $^{14}$N quadrupole parameters were also observed in ESEEM spectra of model systems [71,72] and of other biological systems, for example in bacterial reaction center [73–75] (Table 2). In these works it was demonstrated that the size of the quadrupole coupling is indicative of a backbone amide that is hydrogen bonded to the spin-carrying center. In the iron–sulfur–proteins such couplings were explained by the presence of hydrogen bonds between backbone amide protons (N–H) and the sulfur atoms of the cluster, which are typical for all iron–sulfur–proteins. Both the bridging (μ-S) sulfurs of the cluster and/or the thiolate (S$^-$) sulfurs (mercaptides) of the cysteine ligands could be involved in such H-bonding of the iron–sulfur cluster.

### 3.5.2. Q-Band ENDOR

Two broad lines with maxima separated by approximately 1 MHz were observed in the $^1$H ENDOR spectra of the reduced holo FB peptide (Fig. 6). These lines were assigned to the β-CH$_2$ protons of cysteines ligating the [4Fe–4S] cluster. The eight β-CH$_2$ protons of all four cysteines ligating the [4Fe–4S] cluster most likely contribute to these lines [70,76,77]. Our assignment is based on the similarity to the previously described cases of β-CH$_2$ protons ligating the cubane [3Fe–4S] cluster in \textit{D. gigas} hydrogenase [70], where a range of CH$_2$ hyperfine couplings of 1.3 to 1.9 MHz was found.

In a separate experiment the [4Fe–4S] clusters were inserted into the apo-FB peptide in 99% deuterium oxide buffer (the pH was adjusted for the deuterium isotope effect). Since the apo peptide is completely unfolded and has no secondary structure in the absence of the iron–sulfur cluster, this should result in an exchange of all amide protons (H) to deuterons (D) prior to iron–sulfur cluster formation.

### Table 2

<table>
<thead>
<tr>
<th>Protein/compound</th>
<th>$\varepsilon^{2qQ}/h$, MHz</th>
<th>$\eta$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-glycinea N(2)</td>
<td>3.03</td>
<td>0.41</td>
<td>[71]</td>
</tr>
<tr>
<td>Tri-glycinex N(2)</td>
<td>3.01</td>
<td>0.48</td>
<td>[71]</td>
</tr>
<tr>
<td>Tri-glycine N(3)</td>
<td>3.08</td>
<td>0.76</td>
<td>[71]</td>
</tr>
<tr>
<td>Polyglycine</td>
<td>3.07</td>
<td>0.76</td>
<td>[72]</td>
</tr>
<tr>
<td>Fumarate reductase Center 1 ([2Fe–2S]$^-$) from \textit{Escherichia coli}</td>
<td>3.30</td>
<td>0.5</td>
<td>[65]</td>
</tr>
<tr>
<td>Bacterial reaction center (Q$_{a^-}$) from \textit{Rhodopseudomonas viridis}</td>
<td>3.20</td>
<td>0.52</td>
<td>[73,74]</td>
</tr>
<tr>
<td>Bacterial reaction center (Q$_{a^-}$) from \textit{Rhodobacter sphaeroides}</td>
<td>3.05</td>
<td>0.54</td>
<td>[73,75]</td>
</tr>
<tr>
<td>holo FB peptide</td>
<td>3.0 ± 0.1</td>
<td>0.70 ± 0.02</td>
<td>This work</td>
</tr>
<tr>
<td>holo FB peptide</td>
<td>3.1 ± 0.1</td>
<td>0.75 ± 0.04</td>
<td>This work</td>
</tr>
</tbody>
</table>

Comparison with holo FA and holo FB peptides.

* $^a$H$_3$N(1)-CH$_2$-CO-N(2)-H-CH$_2$-COO$^-$.

* $^b$H$_3$N(1)-CH$_2$-CO-N(2)-H-CH$_2$-CO-NO(3)-H-CH$_2$-COO$^-$.

![Figure 5](image_url)

**Fig. 5.** (A) X-band pulse EPR spectrum of the reduced holo FB peptide. The position of the maximum ($g_2 = 1.93$) of the EPR, where ESEEM was measured, is marked with an arrow. Note that the small signal at 3480 G is due to excess of the sodium dithionite reductant. (B and C) The ESEEM spectra (solid lines) and simulations (dashed lines) of reduced holo FA and holo FB peptides. Simulation parameters for holo FA peptide: $A_{iso} = 0.90 ± 0.05$ MHz, $\varepsilon^{2qQ}$/h = 3.0 ± 0.1 MHz, $\eta = 0.70 ± 0.02$ and for holo FB peptide: $A_{iso} = 0.70 ± 0.07$ MHz, $\varepsilon^{2qQ}$/h = 3.1 ± 0.1 MHz, $\eta = 0.75 ± 0.04$.

![Figure 6](image_url)

**Fig. 6.** (A) X-band pulse EPR spectrum of the reduced holo FB peptide. The position of the maximum ($g_2 = 1.93$) of the EPR, where ESEEM was measured, is marked with an arrow. Note that the small signal at 3480 G is due to excess of the sodium dithionite reductant. (B and C) The ESEEM spectra (solid lines) and simulations (dashed lines) of reduced holo FA and holo FB peptides. Simulation parameters for holo FA peptide: $A_{iso} = 0.90 ± 0.05$ MHz, $\varepsilon^{2qQ}$/h = 3.0 ± 0.1 MHz, $\eta = 0.70 ± 0.02$ and for holo FB peptide: $A_{iso} = 0.70 ± 0.07$ MHz, $\varepsilon^{2qQ}$/h = 3.1 ± 0.1 MHz, $\eta = 0.75 ± 0.04$. (This work)

In a separate experiment the [4Fe–4S] clusters were inserted into the apo-FB peptide in 99% deuterium oxide buffer (the pH was adjusted for the deuterium isotope effect). Since the apo peptide is completely unfolded and has no secondary structure in the absence of the iron–sulfur cluster, this should result in an exchange of all amide protons (H) to deuterons (D) prior to iron–sulfur cluster formation. In
the $^1$H ENDOR spectra no effect on the lines with couplings larger than 1 MHz was detected upon deuteration (Fig. 6B). This corroborates our assignment of the strongly coupled lines to non-exchangeable $\beta$-CH$_2$ protons of cysteines ligating the [4Fe-4S] cluster. The amplitude of the signal at the $^1$H Larmor frequency (matrix line) considerably decreased. The complementary deuterium ENDOR spectrum of the same holo F$_8$ sample was also recorded (Fig. 6C). The $^3$H ENDOR line is broad and unresolved. The width of the line (0.7 MHz) is significantly larger than that expected for matrix (bulk solvent) deuterons. This is due to the anisotropic hyperfine and quadrupole interactions [78,79].

Most likely the $^3$H ENDOR line originates from the structural hydrogen bond(s) between the backbone ND$_2$ group(s) and sulfur atom(s) of the [4Fe-4S] cluster. Hydrogen bonds between backbone nitrogens and the sulfur atoms of the iron–sulfur cluster are typical structural features of the ferredoxin binding sites in proteins [80]. This agrees well with the ESEEM data on the holo F$_8$ and holo F$_9$ peptides, discussed above. The EPR amplitude of the reduced holo F$_8$ peptide at Q-band was found to be considerably smaller and insufficient for observation of the $^1$H ENDOR spectra on this sample.

3.6. Oxidation–reduction potential of the [4Fe–4S] clusters bound to the F$_8$ and F$_9$ peptides

The redox potential of the holo F$_8$ and the holo F$_9$ peptides was determined by potentiometric titration (Fig. 7). Two different methods were used for detection of the gradual sample reduction during the titration. The first method relies on detection of the bleaching of the broad S→Fe charge-transfer band in the UV/Vis spectrum (around 400 nm) upon reduction of the iron–sulfur clusters. The second relies on EPR detection of the [4Fe–4S] cluster whose amount increases upon gradual reduction of the sample. In both cases, chemical reduction is performed by the addition of sodium dithionite at pH = 10 under strictly anaerobic conditions. Both methods give values identical within the error of the measurement. We estimated the redox potentials of holo F$_8$ to be $E_m = -0.44 \pm 0.03$ V and of holo F$_9$ to be $-0.47 \pm 0.03$ V.

3.7. Binding of the holo F$_8$ and holo F$_9$ peptides to the P700-F$_x$ core

The P700-F$_x$ core is a PS I preparation where the stromal subunits PsaC, PsaD and PsaE as well as the terminal [4Fe–4S] clusters F$_8$ and F$_9$ were removed by treatment of the PS I complex with chaotropic agents [81–83]. It is well-known that PsaC can be rebound to the P700-F$_x$ core, re-establishing electron transfer to F$_8$/F$_9$ [34,37,84,85] and the ability of PS I to reduce ferredoxin/flavodoxin upon a flash of light [56,86,87]. Since our peptides model the [4Fe–4S] clusters F$_8$ and F$_9$ of the PsaC subunit of PS I, we tested if each of them would be able to bind to the P700-F$_x$ core and possibly participate in the light-induced electron transfer in PS I.

In isolated PS I, and complexes derived from it (such as the P700-F$_x$ and other cores), photoinduced charge separation is followed by charge recombination between P700$^+$ and the terminal electron acceptor. This process could be monitored by measuring either the decay of the photoinduced absorbance change of the terminal acceptor, or the decay of the P700$^{+}$ after initiation of electron transfer by a laser flash. The latter approach has been used in this work both for monitoring the preparation of P700-F$_x$ cores and for the investigation of the binding of the model peptides to these cores.

It was shown previously that the kinetics of backreaction in isolated PS I complexes is characterized by two main phases with lifetimes ($\tau$) of ca. 30 and 100 ms [88,89] (reviewed in [90]). Note, that the backreaction lifetimes reported here are species and preparation dependent. In P700-F$_x$ cores the terminal acceptors F$_8$ and F$_9$ are missing, consequently a faster reduction of P700$^+$ is observed with a backreaction occurring with $\tau$ of 10 and 200 µs, and 1.5–5 ms [48,85,91]. The P700–A$_1$ cores, isolated from PS I, additionally lack the interpolypeptide [4Fe–4S] cluster F$_9$ [92]. Here the backreaction occurs with $\tau$ of ca. 10 and 200 µs and is attributed to charge recombination between P700$^+$ and A$_{1}^{+}$ [90,93,94]. In P700–F$_x$ cores the
backreactions in the μs time range are also attributed to charge recombination between P700\(^{−}\) and A1\(^{−}\) and those in the ms time range to charge recombination between P700\(^{−}\) and FX [85-90,93,94]. Gong et al. [85] showed that the contribution of the P700\(^{−}\)-A1\(^{−}\) charge recombination in part of the P700-FX cores comes from the inhibition of electron transfer between A1 and FX in the absence of PsaC. Thus the presence of this phase in charge recombination is not due to the partial destruction of FX during the isolation procedure and formation of some P700-A1 cores instead of P700-FX cores.

We isolated P700-FX complexes from Synechococcus sp. PCC 7002 (see Materials and methods section). Either the holo Fx or holo Fs peptide was anaerobically incubated overnight with P700-FX cores. The excess of peptide was removed by repeated ultrafiltration. Note that we attempted to individually bind the holo Fs or holo Fs peptides to P700-FX cores. Charge recombination kinetics of the P700\(^{−}\) reduction in the P700-FX cores, and P700-FX complexes with bound holo Fs or holo Fs peptides are shown in Fig. 8. Kinetic analysis of the P700\(^{−}\) reduction in these preparations is summarized in Table 3.

Based on previous investigations of P700-FX cores, the backreactions with lifetimes of 11 and 260 μs are attributed to recombination between P700\(^{−}\) and A1\(^{−}\) and the backreaction with lifetimes of 1.5–5 ms to charge recombination between P700\(^{−}\) and FX\(^{−}\) [48,85,90,93,94]. The longer lived kinetic phases (>5 ms) were detected only in P700-FX cores incubated with holo Fs or holo Fs peptides. Their appearance coincides with the decrease in the contribution of the kinetic phases belonging to the P700\(^{−}\)-A1\(^{−}\) charge recombination in P700-FX core preparation.

Both peptides change the backreaction kinetics of P700-FX cores irreversibly, since excessive washing does not recover the original backreaction kinetics (Materials and methods). Such changes in kinetics show that both holo Fs and holo Fs peptides can bind to P700-FX cores and possibly participate in the light-induced electron transfer in PS I. An alternative explanation could be that our model peptides irreversibly change the environment of the interpolypeptide [4Fe-4S] cluster Fs leading to changes in the backreaction times. However, we believe that the latter explanation is rather unlikely.

The efficiency of holo Fs binding to P700-FX cores is higher (18%) than that of holo Fs (10%). This is not surprising since, unlike Fs, the Fs peptide sequence contains two key residues (Lys 51 and Arg 52) that are responsible for binding of Psac to P700-FX core [23,36] (Fig. 1).

In summary, according to our optical data both holo Fs and holo Fs irreversibly bind to P700-FX cores and change the charge recombination kinetics. This implies that they could participate in the light-induced electron transfer.

### 4. Discussion

#### 4.1. Comparison of model peptides Fs and Fb with previous peptide-based models of [4Fe-4S] proteins

Two alternative modelling approaches were developed in the past few years to model [4Fe-4S] clusters in proteins. Gibney et al. showed that low-potential [4Fe-4S] clusters could be inserted into a sixteen amino acid synthetic polypeptide, whose sequence was derived from ferredoxin I from *Peptococcus aerogenes* [8]. Alternatively, a high-potential iron–sulfur cluster was successfully introduced into the hydrophobic core of thioredoxin from *E. coli* [12]. The former approach is close to the one we used in our work. Mullolland et al. focused on understanding the fundamental ligand requirements for the successful binding of low-potential [4Fe-4S] clusters by a sixteen amino acid maquettes [9]. In the follow-up work a minimal peptide, seven amino acids in length, which was capable of successful binding a low-potential [4Fe-4S] cluster was developed and the role of non-ligating amino acids in such maquettes was investigated [10].

The sequences of the synthetic peptides Fs and Fb, studied here satisfy the design rules established previously for model peptides [8–10]. Both peptides contain three appropriately spaced Cys residues, the second amino acid in the CxxCxxC motif is Val in Fs and Leu in Fs, the third position is occupied by Gly in both peptides. The fifth position is occupied by Lys in Fs and Thr in Fs. These two amino acids are the second and the third most prevalent at this position among previously studied iron–sulfur proteins [10]. An Arg residue that is found in Fs at the sixth position within the CxxCxxC binding motif is the second most prominent amino acid among all studied iron–sulfur proteins, while Gln found in Fs is also relatively common [10]. The fourth ligand to the iron–sulfur clusters is provided by Cys, which is part of the loop introduced by the KPECPW sequence, similarly to the ferredoxin maquette, FdM-Pa, described previously [9]. Note that in the binding site of Fs Lys at the fifth and Arg at the sixth positions are well conserved in Psac sequences from different organisms since they play a key role in binding of Psac to the P700-Fx core of Ps i (Fig. 1A) [23,36].

The iron–sulfur cluster insertion procedure used here [35,37,38] is very similar to the one employed in previous studies of synthetic peptides modelling binding sites of [4Fe-4S] clusters [8–10], and is based on the original work of Lovenberg et al. [40]. Combination of the data obtained by optical, EPR and Mössbauer spectroscopies conclusively proves that low-potential [4Fe-4S] clusters are incorporated into both of our model peptides Fs and Fb (see Results and Figs. 2–4).

#### 4.2. Comparison of CW and pulse EPR and Mössbauer spectroscopic data obtained on holo Fs and holo Fs peptides and the Psac subunit of PS I

This work focuses on the investigation of the properties of the [4Fe-4S] clusters bound to maquettes, which are compared to the properties of the [4Fe-4S] clusters bound to Psac, investigated in parallel by identical methods. Wild-type Psac contains two [4Fe-4S] clusters and, upon reduction, an “interaction spectrum” is observed reflecting magnetic interaction of two \(S = 1/2\) paramagnetic centers. In order to circumvent this problem the EPR spectra of reduced holo Fs and holo Fs peptides were compared with the spectra measured on two variants of the Psac in the reduced state.

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2 The detection of holo Fs and holo Fs bound to P700-Fs core by EPR at low temperature was attempted, but it was not successful. In these experiments PS I samples, typically of 0.5–0.6 mg/ml Chl concentration, are illuminated inside the EPR cavity (see [94] for a recent example) for accumulation of the P700\(^{−}\)-holo Fs or P700\(^{−}\)-holo Fs states. In our case these experiments most likely did not succeed due to only partial binding of the model peptides to P700-Fs cores, which in both cases is under 20%. This leads to a low concentration of P700\(^{−}\)-holo Fs or P700\(^{−}\)-holo Fs states. Illumination of more concentrated sample is problematic due to the high optical density of the sample.

3 The efficiency of iron–sulfur cluster reconstitution was found to be 24% for Fs and 12% for Fb. Note that the method used here for this calculation is very different from the one used before [8–10]. In the previous work, only the efficiency of iron–sulfur cluster reconstitution relative to the ferredoxin maquette (FdM) peptide was reported for synthetic peptides other than FdM [8–10]. For FdM it was reported to be >60%, based on spin-quantitation of the EPR signal of the dithionite-reduced [4Fe-4S] cluster [8], but unfortunately no details of this procedure were reported. Thus differences in procedure could account for discrepancies in the iron–sulfur cluster reconstitution efficiency of our and previously obtained data.
We use the C13G C33S and C50G C33S mutants of PsaC [53–55,86] for comparison to holo F\(_a\) and holo F\(_b\) peptides, respectively. The C13G C33S variant of PsaC lacks the second cysteine ligand in the binding site of the iron–sulfur cluster F\(_a\) (C13). Similarly, the C50G C33S variant of PsaC lacks the second cysteine ligand in the binding site of the iron–sulfur cluster F\(_b\) (C50). C33 in not involved in ligation of either F\(_a\) or F\(_b\) [4Fe–4S] clusters in PsaC, thus the C33S variant is used to reduce non-specific iron binding during insertion of the clusters [57]. Both C13G and C50G mutants are “rescued” by an external thiolate ligand which provides the fourth ligand to the iron–sulfur cluster. It is derived from 2-mercaptoethanol, which is present in the reaction mixture during iron–sulfur cluster reconstitution into the apo protein [53–55,86]. Interestingly, in the modified site the [4Fe–4S] cluster becomes \(S\geq3/2\) and appears to have no magnetic interaction with the second iron–sulfur cluster in the protein [55]. This allows the observation of the EPR signal, around \(g\geq2\), of the unmodified \(S=1/2\) iron–sulfur–cluster F\(_a\) in the C13G C33S variant and F\(_b\) in the C50G C33S variant. The EPR signal of the mutated cluster is observed at \(g\)-values larger than 4.5 in both variants, \(S\geq3/2\) [53–55].

The EPR spectra, around \(g=2\), of both variants of PsaC are very similar with \(g_1=2.04\), \(g_2=1.93\) and \(g_3=1.90\) for the F\(_a\) cluster (C13G C33S variant) and \(g_1=2.05\), \(g_2=1.93\) and \(g_3=1.89\) for the F\(_b\) cluster (C50G C33S variant), the former one having a broader linewidth [53,55]). These values are nearly identical to those obtained for the reduced holo F\(_a\) and holo F\(_b\) peptides. Similarly, the reduced holo F\(_a\) peptide has a larger line width. These values are also very similar to the previously reported ones for model peptides binding iron–sulfur–clusters, where \(g_1=2.05\), \(g_2=1.93\) and \(g_3=1.89\) was found [8–11].

Previously, pulse EPR methods, such as ESEEM [95,96] and ENDOR [97,98], have been used successfully to investigate the local structure of paramagnetic centers in biological samples [99–102]. Up to now such methods have not yet been applied to peptide-based models of iron–sulfur–clusters. ESEEM spectra recorded on the reduced holo F\(_a\), holo F\(_b\) peptides (Fig. 5) and the C13G C33C variant of PsaC were consistent with structural hydrogen bond(s) between sulfur atom(s) and amide backbone proton(s) are present in the model peptides as well as in PsaC. The deuterium ENDOR spectrum obtained on reduced holo F\(_a\) peptide further supports this finding. Hydrogen bonding between backbone amide protons and sulfur atoms of the iron–sulfur cluster is a well-known structural feature of low-potential iron-sulfur clusters and shows that our peptides are realistic models for ferredoxins and for the PsaC subunit of PS I.

The Mössbauer spectra of the holo F\(_a\) and holo F\(_b\) peptides, both in the oxidized and reduced forms, were also compared to similar spectra of the C13G C33S and C50G C33S mutants of PsaC (Table 1). All the data indicate the presence of [4Fe–4S]\(^{2+}\) clusters in the oxidized samples and [4Fe–4S]\(^{1+}\) clusters in the reduced samples. Holo F\(_a\) and holo F\(_b\) peptides, to date, are only the second peptide-based models containing [4Fe–4S] clusters that were investigated by Mössbauer spectroscopy and the only ones investigated in both oxidized and reduced states. Previously helix–loop–helix peptides containing [4Fe–4S] and a bridged assembly [Ni\(^{2+}\)-(µ-S–Cys)]-[4Fe–4S] [17] were investigated by Mössbauer spectroscopy, confirming the presence of a [4Fe–4S] cluster in the oxidized (2+) state in both models.

### 4.3. Redox potentials of the model F\(_a\) and F\(_b\) peptides

Most of the previously designed models contained the Clostridial ferredoxin consensus iron–sulfur–cluster binding site, including non-ligand amino acids [8,9,11]. These model peptides have a potential of \(-0.350\) V [8,9] or even higher (\(-0.289\) V) [11]. This lies within the broad range of potentials for low-potential iron–sulfur–proteins in general [2]. However, these values are higher and different from the previously reported midpoint potentials of ferredoxins from \(C.\) pasteurianum (\(-0.403\) V), \(C.\) acidituniurici (\(-0.434\) V) and \(P.\) aerogenes (\(-0.427\) V) [103,104]. The lowest redox potential, which is reported for a model peptide containing a [4Fe–4S]\(^{2+/1+}\) cluster so far, is \(-0.422\) V [13]. It was found for the 4-α-helix bundle containing the binding site of an interpolyprotein [4Fe–4S] cluster F\(_b\) of PS I [113]. Note, that the binding motif of F\(_b\) is drastically different from the Clostridial ferredoxin binding site.

Both model peptides described in our work contain the Clostridial ferredoxin consensus CxxCxXC [4Fe–4S] cluster binding motif. We determined the redox potential of holo F\(_a\) to be \(-0.44\pm0.03\) V and of holo F\(_b\) to be \(-0.47\pm0.03\) V. Note that our data are an upper estimate and the actual potential could be even lower. Also, while the error of our measurement is quite significant the potentials that we found are at least 0.05 to 0.09 V more negative than those previously published for short peptides modelling Clostridial ferredoxins [8,9] and are very close to the data obtained for the only reported model for the [4Fe–4S] cluster F\(_b\) [13]. More importantly, these potentials are very close to typical midpoint redox potentials found for [4Fe–4S] clusters in bacterial ferredoxins. They correlate well with the redox potentials of \(-0.465\) V and \(-0.440\) V that were found for F\(_a\) and F\(_b\), respectively, in PS I [31]. This underlines the biological relevance of our models.

In the ESEEM and \(^{2}H\) ENDOR spectra (Figs. 5 and 6) we detected hydrogen bond(s) between sulfur atom(s) of the cluster and the backbone amide proton(s). This is known to stabilize the reduced (1+) state of [4Fe–4S] clusters [80,104] which lowers the reduction potential. Solvent accessibility is another feature that contributes to the decrease of the reduction potential of [4Fe–4S] clusters [80,104]. The broadness of the ENDOR line belonging to \(\mu-\) CH\(_2\) protons of cysteines ligating the [4Fe–4S] cluster in the holo F\(_a\) peptide indicates flexibility of the polypeptide chain around the metal center, thus allowing virtually unhindered, i.e. easy access of water molecules to the cluster.

### 4.4. Binding of the holo F\(_a\) and holo F\(_b\) models to P700–F\(_X\) cores

After successful modelling of the iron–sulfur–clusters F\(_a\) and F\(_b\) bound to the PS I subunit PsaC, it is important to determine if each of our models can bind to PS I (P700–F\(_X\) core) and possibly participate in the light-initiated electron transfer in PS I. This would open a way for practical use of these models as building blocks in the construction of biomimetic systems that can be used for energy production.

As described above, the F\(_b\) peptide sequence contains two key residues (Lys 51 and Arg 52, Fig. 1) that are crucial for binding of PsaC to the P700–F\(_X\) core via the formation of salt bridges with amino acids on the PsaA and PsaB subunits [23,36]. In contrast, the F\(_a\) peptide does not contain such residues. Therefore, it is quite surprising that holo F\(_a\) is capable of binding to the PS I cores at all. However, as expected, binding of holo F\(_a\) is more efficient (18% for holo F\(_b\) and 10% for holo F\(_a\)). It is interesting to compare our data with rebinding of PsaC to P700–F\(_X\) cores in vitro.

The PsaC deletion mutant was constructed in Synecchocystis sp. PCC 6803 [85,105], rendering cells that were incapable of photoautotrophic growth. PS I is assembled in this mutant. However, it lacks the stromal subunits PsaC, PsaD and PsaE as well as the PsaC-bound terminal [4Fe–4S] clusters F\(_X\) and F\(_a\). Thus, the in vivo P700–F\(_X\) core is formed, which could be isolated from these cells without chemical treatment of the PS I complex. It was shown that recombinant PsaC could be rebound to these P700–F\(_X\) cores [56,85]. However, a reduced efficiency of rebinding was reported: 43% [56] and 32% [85]. The rebinding of recombinant PsaC to P700–F\(_X\) cores, prepared by urea treatment of PS I, was reported to occur with 41% efficiency [86]. In summary, the results obtained on rebinding PsaC in vitro are compatible to our results on binding of the small peptides holo F\(_b\) (18%) and holo F\(_a\) (10%) to P700–F\(_X\) cores.

Binding of holo F\(_a\) and holo F\(_b\) to P700–F\(_X\) cores isolated from two different cyanobacterial species Synecchocystis sp. PCC 6803 (data not shown) and Synecchococcus sp. PCC 7002 implies that such binding is a general quality rather than a species specific effect.
An important point is that our evidence for binding of holo F₈ and holo F₆ to P700-F₃ cores are based solely on the irreversible change of the charge recombination in P700-F₆ cores upon incubation with either holo F₈ or holo F₆ peptides. We are continuing our research and are looking for further evidence of this.

Overall, it is quite exciting that the small peptides can bind to PS I cores and participate in the light-induced electron transfer. This can be used for chemical attachment of different molecules to the acceptor side of PS I. It could be done, for example, via attaching a chemical rescue ligand to the [4Fe–4S] cluster, as described previously [55], and/or by directly engineering proper amino acids in the binding sequence of the custom designed peptides. These findings could have implications for the construction of new alternative energy sources, e.g. for light-driven hydrogen production, specifically, if similar peptide models will be used to attach [FeFe] or [NiFe] hydrogenases directly to PS I [106].

5. Summary and conclusions

Two sixteen amino acid peptides modelling the binding sites of the [4Fe–4S] clusters F₈ and F₆ of photosystem I were prepared. By optical-, EPR- and Mössbauer spectroscopies it has been conclusively proven that holo F₈ and holo F₆ model peptides bind [4Fe–4S]²⁺/¹⁺ clusters. The midpoint reduction potential of holo F₆ was determined to be −0.44 ± 0.03 V and that of holo F₈ to be −0.47 ± 0.03 V. These values are considerably lower than the ones previously reported for similar model systems and are very close to the ones of F₈ and F₆ in photosystem I. By ESEEM and ¹H ENDOR spectroscopies it was shown that the iron–sulfur clusters in both peptides are hydrogen bonded via amide of the peptide backbone. This demonstrates the structural integrity of our models and could, in part, explain the relatively low reduction potential found for holo F₈ and holo F₆. By optical spectroscopy we have found that both model peptides can bind to the P700-F₆ core. Our data suggest that bound peptides could serve as electron acceptors during light-initiated electron transfer. The ability of the model peptides to participate in the light-induced electron transfer can open new avenues for the construction of hybrid biological/chemical systems for conversion of light into chemical energy.

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