In bacteria, riboflavin phosphorylation and subsequent conversion of FMN into FAD are carried out by FAD synthetase, a single bifunctional enzyme. Both reactions require ATP and Mg²⁺. The N-terminal domain of FAD synthetase appears to be responsible for the adenylyltransferase activity, while the C-terminal domain would be in charge of the kinase activity. Binding to Corynebacterium ammoniagenes FAD synthetase of its products and substrates, as well as of several analogues, is analysed. Binding parameters for adenine nucleotides to each one of the two adenine nucleotide sites are reported. In addition, it is demonstrated for the first time that the enzyme presents two independent flavin sites, each one related with one of the enzymatic activities. The binding parameters of flavins to these sites are also provided. The presence of Mg²⁺ and of both adenine nucleotides and flavins cooperatively modulates the interaction parameters for the other ligands. Our data also suggest that during its double catalytic cycle FAD synthetase must suffer conformational changes induced by adenine nucleotide-Mg²⁺ or flavin binding. They might include not only rearrangement of the different protein loops, but also alternative conformations between domains.

Flavoproteins are involved in a large variety of biological processes (DNA repair, apoptosis, oxidative phosphorylation, photosynthesis, etc) and require the riboflavin-derived redox cofactors FMN (flavin mononucleotide or riboflavin 5′-phosphate) or FAD (flavin and adenine dinucleotide) for their function. In vivo, riboflavin (RF) is converted into FMN first and then into FAD via the sequential action of an ATP:riboflavin kinase (RFK, (EC 2.7.1.26)) and an ATP:FMN adenylyltransferase (EC 2.7.7.2) (Figure 1A). Eukaryotes generally use two different enzymes for FMN and FAD production (1-7), whereas most prokaryotes depend on a single bifunctional enzyme, the FAD synthetase (FADS) (8,9).

The two catalytic cycles of FADS involve the binding of 2 ATP, 1 RF and 1 FMN molecules as substrates and the production of 1 ADP, 1 PPi, 1 FMN and 1 FAD. The proposed pathway for the phosphorylation reaction would be for RF to bind before ATP, while ADP releases prior FMN (10). In the adenylylation process, FMN is proposed to bind after ATP and the PPi to be released preceding FAD (10). The two enzymatic activities differ in their specificity for divalent cations, optimal pH and temperature (8,11,12). The presence of Mg²⁺ improves the turnover of both processes but, while low concentrations (<1 mM) enhance the kinase activity, much larger concentrations (~10 mM) are required for maximal FAD production (8). These studies indicated the presence of two independent ATP-binding sites, one at the RF-phosphorylation site and one at the FMN-adenylylation site, but a single pocket was proposed to allocate the isoalloxazine-ribityl moieties of both substrates, RF and FMN, in the two reactions (10).

The only structure reported for an FADS is that of Thermotoga maritima (TmFADS), both free and in complex with several substrates (13,14). One of these structures shows simultaneous binding of AMP in the N-terminal domain, and ADP and FMN in the C-terminal. Thus, the protein folds in two almost independent domains, each one hosting one ATP-binding site, while only a flavin-binding site, located in the C-terminal domain, was detected (14). However, in the C-terminal domain the ribityl and phosphate of the flavin are not placed in the putative active site. This is
probably due to the disorder in the protein regions hosting the flavin. The C-terminal domain shows structural homology with *H. sapiens* and *S. pombe* RFKs (14-17) and can catalyse the phosphorylation of RF (15). The N-terminal region presents remote similarities with nucleotidylyltransferases (NTs) (18), but it does not appear to be self-sufficient to transform FMN into FAD (15). The TmFADS structure shows a large distance between the reported flavin binding site and the adenyllylation site (14). Despite the asymmetric unit is a dimer, it does not appear functional. These observations, together with the fact that the FMN produced in the phosphorylation process has to be released before rebinding as substrate for the second reaction (10), make it logical to propose the presence of a second flavin binding site, located in the N-terminal domain. The structural model of FADS from Corynebacterium ammoniagenes (CaFADS) shows the main structural arrangements present in TmFADS (13,14), and it also shows the disposition of some loops and a 3_{10}-helix at the C-terminal domain that are missed in the TmFADS structure. Additionally, the C-terminal domain of CaFADS showed an insertion around residue 230 with regard to TmFADS. This model also suggests the presence of a novel putative second flavin binding site in the N-terminal domain (15) (Figure 1B). This site would be located in the proximity of the adenine nucleotide binding site and might bind FMN for adenyllylation.

The present study provides with a detailed thermodynamic analysis of the binding of adenine nucleotide and flavin ligands to CaFADS and to its individually cloned C-terminus domain. It confirms the presence of two flavin binding sites in CaFADS and allows assigning affinities to the different flavin and adenine nucleotide binding sites.

**EXPERIMENTAL PROCEDURES**

Cloning, over-expression and purification of CaFADS and its C-terminal domain- WT CaFADS was cloned, over-expressed and purified as previously described (15). The separately cloned C-terminal domain (∆(1-182)FADS) was purified using a similar protocol, replacing the DEAE-cellulose chromatography with a Superose 12 gel-filtration (GE Healthcare).

**Spectral analysis**- UV-Vis spectra were recorded on a Cary-100 spectrophotometer. To determine the extinction coefficient (ε) of CaFADS in 50 mM Tris/HCl, pH 8.0 and 20 mM sodium phosphate, pH 7.0, the UV-Vis absorbance spectrum of the protein was recorded in each buffer. The samples were subsequently diluted with 7.5 M guanidinium hydrochloride in 20 mM sodium phosphate, pH 6.5, to 6 M guanidinium hydrochloride. The absorbance spectra were again recorded. Protein concentration was calculated using the theoretical ε_{280nm} for the denatured protein (19). ε_{280nm} in each buffer was obtained from the initial spectra. For ∆(1-182)FADS, the theoretical ε_{280nm} of 14 mM⁻¹ cm⁻¹ was used (19).

Difference spectroscopy measurements were carried out in 50 mM Tris/HCl, pH 8.0. The reference cuvette, containing buffer, and the sample cuvette, containing 5-6 µM FADS (3 µM for titration with RF), were stepwise titrated with aliquots of 1-10 µl of the corresponding flavin solutions (~180 µM RF, ~750-1500 µM FMN and ~450-600 µM FAD). For the measurements in the presence of AMPPNP, a non-hydrolysable ATP analogue, both cuvettes contained 1 mM AMPPNP and 10 mM MgCl₂. Dissociation constants and difference extinction coefficients were obtained by non-linear regression fit of the experimental data to the theoretical equation for a 1:N stoichiometric complex (20):

\[
\Delta\text{Abs} = \frac{\Delta\varepsilon l \left( [FADS] + [L] + K_d \right)}{2N \left( -\sqrt{[FADS] + [L] + K_d} - 4[FADS][L] \right)}
\]

where Δε is the change in the flavin extinction coefficient upon ligand binding to FADS, \( l \) is the cuvette path length, \([FADS]\) and \([L]\) are the total concentration of FADS and flavin, respectively, \( K_d \) is the dissociation constant and \( N \) is the number of flavin binding sites in FADS. Errors estimated for \( K_d \) and \( \Delta\varepsilon \) were ± 15 %.

CD spectra were recorded in a Chirascan spectropolarimeter (Applied Photophysics Ltd.) at 5 °C in the far-UV (7 µM FADS in 5 mM sodium phosphate, pH 7.0, 0.1 cm cuvette) and in the near-UV (20 µM FADS in 20 mM sodium phosphate, pH 7.0, 0.4 cm cuvette). Near-UV CD spectra were also recorded at 20 °C with saturating concentrations of ATP, ADP and/or FMN, at both 0 and 10 mM MgCl₂.

Fluorescence emission spectra were recorded in an Aminco-Bowman Series 2 spectrometer in
20 mM sodium phosphate pH 7.0, at 25 °C, exciting the protein aromatic residues at 280 nm.

Differential scanning calorimetry (DSC)- The heat capacity of FADS (ΔC_p) was measured as a function of temperature at pH 7.0 with a VP-DSC microcalorimeter (MicroCal LLC). Thermal denaturation scans were performed with 8 and 24 µM degassed FADS solutions in 20 mM sodium phosphate, pH 7.0, at a scanning rate of 1°C/min from 10 to 100 °C. Reversibility of the unfolding process was checked by sample reheating after cooling inside the calorimetric cell. The unfolding of FADS was not reversible and, therefore, only a van’t Hoff model-independent analysis was performed (21,22).

High sensitivity isothermal titration calorimetry (ITC)- Measurements were carried out using a high-precision VP-ITC system (MicroCal LLC). Typically, a ~300 µM FAD or FMN solution, or a 300-500 µM ADP, ATP or AMP-PNP solution was used to titrate ~10-20 µM FADS. Due to the low solubility of Lumiflavin (LF, a RF analogue lacking the ribityl chain) and RF, FADS solutions of ~5 µM and ~10 µM were titrated with LF and RF ~90 µM and ~175 µM, respectively. Both the ligand and FADS were dissolved in the same buffer (20 mM phosphate, pH 7.0 with variable MgCl2 concentrations: 0, 0.8 or 10 mM) and degassed. This buffer was selected because of its low ionisation enthalpy, which prevents any buffer influence on the observed enthalpy of binding due to possible de/protonation events. Titrations of FADS:nucleotide or FADS:FAD complexes were carried out by stepwise injections of the ligand into a mixture of FADS and a saturating concentration of the adenine nucleotide (400-500 µM) and/or FAD (80-100 µM). Each titration was initiated by a 4 µl injection, followed by 25-28 stepwise injections of 10 µl. The heat evolved after each ligand injection was obtained from the integral of the calorimetric signal. The heat due to the binding reaction was obtained as the difference between the heat of reaction and the corresponding heat of dilution, the latter estimated as a constant heat throughout the experiment and included as an adjustable parameter in the analysis. The association constant (K_a), the enthalpy change (ΔH) and the stoichiometry (N) were obtained through non-linear regression of the experimental data to a model for one or two independent binding sites implemented in Origin 7.0. The dissociation constant (K_d), the free energy change (ΔG), and the entropy change (ΔS) were obtained from basic thermodynamic relationships. Usually, when N=2 there was not enough information in the titrations for using a model with two different binding sites and averaged binding parameters are given. The estimated error was ±15% in K_d and ± 0.3 kcal/mol in ΔH and –TΔS.

To analyse a possible interaction between FADS and Mg^{2+}, 20 µM FADS was titrated with a 300 µM MgCl2 solution. An additional titration of FADS in the presence of 40 µM EDTA with 900 µM MgCl2 was carried out to exclude putative Mg^{2+} binding sites in FADS already being loaded. Additionally, a solution of 40 µM EDTA was titrated with 900 µM MgCl2 to obtain the parameters for the EDTA-Mg^{2+} interaction. Finally, ITC experiments involving the dilution of 440 µM FADS into 20 mM sodium phosphate, pH 7.0, buffer were also performed to test the enzyme oligomerisation state (23,24).

RESULTS

Spectral properties of CaFADS- CaFADS presented an UV-Vis maximum at 279 nm. The ε_{279nm} was 28.1 mM^{-1}cm^{-1} in 50 mM Tris/HCl, pH 8.0 and 25.6 mM^{-1}cm^{-1} in 20 mM sodium phosphate, pH 7.0. The far-UV CD spectrum of CaFADS showed a negative-positive couplet (208-191 nm) typical of a secondary α-helix structure. The negative band around 222 nm, also typical of α-helix, appeared like a shoulder (Figure 2A), probably due to an important content of β-sheet (Figure 1B). The near-UV CD spectrum (Figure 2A), was sensible to substrate binding. Changes were more evident in the simultaneous presence of FMN, ADP or ATP, and Mg^{2+}, and suggest that binding takes place by influencing the environment of aromatic residues. Excitation of CaFADS at 280 nm produced a fluorescence emission band centred at 330 nm, indicating that, in general, the Trp residues of the protein are contained in an apolar environment.

Binding of flavins to FADS followed by differential spectroscopy- Titration of FADS with RF, FMN and FAD produced the appearance of Vis-difference spectra (Figure 2B), indicating changes in the dielectric environment of the isalloxazine upon interaction with FADS. While titration with RF or FMN produced similar difference spectra,
The magnitude of the difference spectra reached saturation (Figure 2D), allowing the determination of $K_d$ and $\Delta C\beta$. Fitting of the data to equation [1] suggested two independent binding sites for RF with an average $K_d$ ($K_{dav}$) of 5.2 μM, while a single binding site was detected for FMN and FAD with $K_{dav}$ of 13.1 and 2.3 μM, respectively. The saturating concentration of AMPPNP-Mg$^{2+}$ produced a considerable increase in $\Delta \epsilon$ when FADS was titrated with RF and, especially, with FMN (Figure 2C). AMPPNP-Mg$^{2+}$ considerably increased the FADS affinity for RF, while decreasing that for FMN ($K_d$ of 0.6 and 90.5 μM, respectively). However, AMPPNP-Mg$^{2+}$ did not affect the FAD interaction (Figure 2).

FADS thermal denaturation followed by DSC- FADS thermal denaturation exhibited two partially overlapping transitions ($T_{mav}$ = 41.1 °C), suggesting that the unfolding processes of the N-terminal and C-terminal domains are not fully cooperative. By direct integration of the signal, a calorimetric $\Delta H$ of 165 kcal/mol and an unfolding $\Delta C_P$ of 4.2 kcal/K·mol were obtained. A statistical analysis of globular proteins indicates that the unfolding $\Delta C_P$ and the unfolding $\Delta H$ at 60 °C scale with protein size according to: $\Delta C_P = 13.9 - N_K$ cal/(K·mol·res) and $\Delta H(60 \degree C) = 0.698 - N_K$ kcal/(mol·res), where $N_K$ is the number of residues in the protein (25). FADS, with 338 residues, has an estimated unfolding $\Delta C_P$ of 4.7 kcal/K·mol and an unfolding $\Delta H(60 \degree C)$ of 236 kcal/mol. They compare well with the experimental values of 4.2 kcal/K·mol and 245 kcal/mol (extrapolation at 60 °C). This suggests that the protein is folded in solution with no significant unstructured regions.

A second cycle of thermal denaturation indicated that the unfolding process was not reversible, the less stable domain being responsible for the non-reversibility. The more stable domain showed a reversible thermal denaturation with a $T_m$ of 43.8 °C. The van’t Hoff analysis provides with a van’t Hoff unfolding $\Delta H$ of 84 kcal/mol. A value of 0.51 for the van’t Hoff-calorimetric $\Delta H$ ratio also indicates that the thermal transition is not fully cooperative and that at least two domains unfold independently. Finally, the lack of effect on $T_{mav}$ upon increasing protein concentration (from 8 μM to 24 μM) indicated that FADS is in the monomeric state at both concentrations, in agreement with the FADS dilution experiment by ITC.

**ITC analysis of the interaction of FADS with Mg$^{2+}$**- Direct titration of the enzyme with MgCl$_2$, did not produce any calorimetric change related to an interaction between FADS and Mg$^{2+}$. This indicates that CaFADS does not show specific binding of free Mg$^{2+}$.

**ITC analysis of the binding of adenine nucleotides to FADS**- The interaction of WT FADS with ATP, ADP and AMPPNP was analysed by ITC at pH 7.0 and 25 °C at different [Mg$^{2+}$] (Figure 3 and Table 1). A single ATP binding site with a $K_d$ of 10 μM was detected for FADS in the absence of Mg$^{2+}$. The presence of this cation produced the appearance of a second ATP binding site and increased the $K_{dav}$, ATP (Table 1). Similarly, FADS showed a single ADP binding site in the absence of Mg$^{2+}$, the affinity 4-times lower than that of ATP (Table 1), and two ADP binding sites in the presence of Mg$^{2+}$ without altering $K_{dav, ADP}$. AMPNP interacted with FADS considerably more weakly than ATP and without major [Mg$^{2+}$] effects. Therefore, AMPPNP is not a suitable ATP analogue for the purpose of this study.

Direct titrations also allowed the determination of the enthalpy and entropy components of the interactions (Figure 4). A favourable enthalpy change drove the interaction of ATP, ADP and AMPPNP with FADS over the unfavourable entropic contribution, although the magnitude was considerable smaller for the latter. Increasing [Mg$^{2+}$] resulted in an slightly less favourable enthalpic binding contribution and a less unfavourable entropic one (Figure 4). However, especially for ADP and AMPPNP, $\Delta G$ remained remarkably insensitive to [Mg$^{2+}$] through entropy/enthalpy compensation.

**ITC analysis of the binding of flavins to FADS**- The interaction of WT FADS with its RF, FMN, and FAD substrates/products, as well as with LF, was also analysed (Figure 5 and Table 1). A single FADS binding site for FMN and FAD was determined, independently of [Mg$^{2+}$], with $K_d$ values in the range of 1-3 μM for both flavins. Binding of FMN and FAD was driven by a large enthalpy change but at high cost in entropy (Figure 6). Increasing [Mg$^{2+}$]
hardly influenced the interaction with FMN, but the interaction with FAD resulted slightly less favoured by the enthalpic contribution and less unfavoured by the entropic one (Figure 6).

Two binding sites were detected when FADS was titrated with RF and LF, both binding only slightly weaker than FMN or FAD (Table 1). Binding of RF and LF was also enthalpically driven with a very small opposing entropic contribution that was favourable in the presence of Mg$^{2+}$ (unlike interactions with FAD and FMN) (Figure 6). This is consistent with the considerably less polar nature of RF and LF.

**ITC analysis of flavin binding to preformed FADS:nucleotide complexes**—A single FAD binding site was unequivocally determined in the presence of saturating concentrations of ADP or AMPPNP (Table 2 and Figure 7). AMPPNP had minor effects on the binding parameters, but the presence of ADP considerably reduced the FADS affinity for FAD at low [Mg$^{2+}$] (Table 2). This reduction in the affinity is the result of considerably less favourable enthalpic and less unfavourable entropic binding contributions (Figures 6 and 8). However, at high [Mg$^{2+}$], binding affinity of FAD to FADS:ADP was similar to that to free FADS due to the less favourable enthalpic contribution being compensated with a favourable binding entropy (Figures 6 and 8).

The preformed FADS:ADP complex was able to bind two FMN molecules. Therefore, the presence of ADP promoted the appearance of a second FMN binding site (Tables 3 and 4). The presence of Mg$^{2+}$ enhanced the FADS:ADP complex affinity for FMN (Table 2). At the highest [Mg$^{2+}$] assayed the independent $K_d$ values for the two FMN binding sites could be determined. Additionally, the simultaneous presence of Mg$^{2+}$ and ADP had an important effect in the enthalpic and entropic binding contributions (Figures 6 and 8). Binding of FMN to the preformed FADS:ADP complex was enthalpically driven with an opposing entropic contribution in the absence of Mg$^{2+}$. However, its presence promoted the enthalpic contribution to become less favourable and transformed the unfavourable entropy into a favourable one, making the interaction stronger. Binding of FMN to the FADS:AMPPNP complex showed similar affinity values to those for the FADS:ADP complex, but a second FMN binding site was not observed (Table 2).

Binding of RF to FADS preloaded with ADP pointed out the presence of a single RF binding site that turned into two sites in the presence of Mg$^{2+}$ (Table 2). The cation also causes a slight increase in the affinity for RF. Two binding sites to FADS:AMPPNP were also detected. The favourable enthalpic and, especially, the unfavourable entropic contributions became considerably less intense upon increasing [Mg$^{2+}$] (Figure 8).

**ITC analysis of competitive ligand binding to preformed FADS complexes**—A set of experiments in the presence of an excess of FAD as competitive ligand was carried out to establish the binding domain of ligands with a single binding site (Table 2). According to previous modelling studies (15) and to our own data, FAD must bind at the N-terminal domain, blocking both the adenine nucleotide and the putative flavin nucleotide binding sites of this domain. Under FAD saturation and lacking Mg$^{2+}$, FMN binding was hardly detected (Table 2). Since in the presence of ADP and 10 mM MgCl$_2$, FADS binds two FMN molecules (Table 2), FADS was simultaneously saturated with ADP and FAD at 10 mM MgCl$_2$ and titrated with FMN. Two FMN binding sites were also detected but the FMN interaction was weaker than in the single ADP presence (Table 2). Two RF binding sites were detected in the FAD:FADS complex with a $K_{dav}^{RF}$ of 13.5 µM (Table 2). Therefore, FAD makes the interaction of FADS with one of the RF molecules slightly weaker.

In the FADS:FAD complex a single binding site for ATP or ADP was again observed (Tables 1 and 2). ATP affinity was not affected, but the presence of FAD considerably hindered ADP binding (Table 2).

**ITC analysis of flavin and adenine nucleotide binding to the C-terminal domain of CaFADS (∆(1-182)FADS)**—The ability of ∆(1-182)FADS to interact with flavins and adenine nucleotides was also studied (Table 3, Figure 9). ∆(1-182)FADS was able to bind a single ATP molecule, but its affinity was weaker than that of WT FADS (Table 3). Despite their smaller magnitude, the enthalpic and entropic contributions at 10 mM MgCl$_2$ showed a profile similar to that of WT FADS (Figures 4 and 9). Noticeably, in the absence of Mg$^{2+}$, ATP biding to ∆(1-182)FADS was driven by a favourable entropic contribution, while the favourable
enthalpic one was considerably smaller than for binding to WT FADS. Binding of ADP to Δ(1-182)FADS was only detected in the presence of Mg\(^{2+}\), with \(K_d\) and enthalpic and entropic binding contributions similar to those for WT FADS (Table 3 and Figures 4 and 9).

No calorimetric profiles were detected upon titration of Δ(1-182)FADS with FAD. Titration with FMN only produced a residual profile that could not be related to a real interaction (Figure 9 left). However, RF was shown to bind at a single site with \(K_d\) of 1.8 and 7.2 \(\mu\)M, at 0 and 10 mM MgCl\(_2\), respectively (Table 3). Binding was entropically driven in the absence of Mg\(^{2+}\) but became enthalpically driven, with an entropic binding contribution still favourable, in its presence (Figure 9). ADP increased the affinity for RF and, particularly, for FMN (Table 3) (Figure 9 left). ADP turned the entropy into an unfavourable contribution for the RF interaction, while the enthalpic one became much more favourable. The FMN interaction was also enthalpically driven, but with a favourable entropic binding contribution of almost the same magnitude (Figure 9).

**DISCUSSION**

This study provides with a thermodynamic characterisation of substrate/product binding to CaFADS and of its individually cloned C-terminus domain, analogous in sequence and structure to the eukaryotic RFKs (15).

Our data confirm that CaFADS holds two different adenine nucleotides binding sites, each of them involved in each one of the enzyme catalytic activities (10). In the absence of Mg\(^{2+}\), the adenine nucleotide binds to a single site (Table 3). Comparison of data for the interaction of the adenine nucleotides with CaFADS, both free and saturated with FAD, and with Δ(1-182)FADS (Tables 1, 2 and 3) allows to distinguish between both sites. Thus, in the absence of Mg\(^{2+}\), ATP only binds to the phosphorylation site at the C-terminal domain, while ADP only binds to the adenylylation site at the N-terminal domain. According to the structural model for CaFADS in complex with its ligands (Figure 1B) (15), an electrostatic repulsion between the highly conserved Asp25 and the \(\gamma\)-phosphate of ATP could prevent ATP binding in the absence of Mg\(^{2+}\), ultimately preventing the adenylyltranferase reaction in the absence of the cation (8). In the C-terminal domain, specificity for ATP versus ADP in the absence of Mg\(^{2+}\) might relate with the localisation of the phosphates, particularly of their acidic groups: when binding ATP, the \(\gamma\)-phosphate acidic groups might be stabilised by the positively charged 195-202 loop, while in the case of ADP the negative charges, located on the \(\beta\)-phosphate, would be surrounded by the negatively charged 268-277 loop.

The presence of Mg\(^{2+}\) induces the appearance of a second adenine nucleotide binding site (Table 1), suggesting that either Mg\(^{2+}\) contributes to reduce the mentioned electrostatic repulsions by participating in the bonding network of the binding pocket or it induces conformational changes in the protein.

Our experimental data do not allow to determine the parameters for each one of the two ATP or ADP binding sites, suggesting the type of interactions with the protein must not differ widely. Particularly, polar contacts are mostly responsible for the protein:nucleotide interaction in both sites (Figure 4), according with the nature of the protein regions where they must bind (15). Nevertheless, the results suggest particular differences. This is in agreement with the selective binding in each of the sites and with the functional and structural differences expected for both sites (10,14,15).

The results for ATP and ADP binding (Table 1, Figure 4) also indicate that the \(\gamma\)-phosphate of ATP is actively involved in the binding at both sites and particularly contributes to the enthalpic and entropic parameters. The different enthalpic and entropic contributions in the binding of ATP to the truncated protein compared to WT FADS (Figures 4 and 9) indicate that the N-terminal region somehow influences nucleotide binding in the C-terminal.

Earlier studies hypothesised on the presence of a single flavin binding site shared by both activities (10,13), but the presence of a second site was recently suggested (15). The present work provides with the experimental evidence of the existence of two independent flavin binding sites in CaFADS. Two binding sites are observed for RF in FADS, while Δ(1-182)FADS binds one RF molecule by itself (Tables 1 and 3). Therefore, one of these sites is situated in the C-terminal domain. The presence of Mg\(^{2+}\) only slightly modulates the RF affinity. \(K_d^{RF}\) values (2.8 \(\mu\)M at 10 mM MgCl\(_2\)).
Mg\(^{2+}\)) are considerably larger than those derived from steady-state kinetic analysis of CaFADS (79 nM) (10). However, they correlate much better with the value described for the rat liver RFK (41 \(\mu\)M) (26). The substantial favourable entropic contribution to RF binding compared to FMN or FAD (Figures 6 and 9), agrees with the hydrophobic isalloxazine binding at the open C-terminal hydrophobic pocket (G223, V224, Y225, F206, P207, F297, L303 and M307) proposed to get closed upon flavin binding (14-16,27). A single binding site in CaFADS is observed for both FMN and FAD. According to the lack of interaction between FAD and \(\Delta(1-182)\)FADS (Table 3), FAD must bind at the N-terminal domain, with the isalloxazine ring putatively allocated by V59, L98, Y106 and F128 (15). The FMN phosphate and a hypothetical negative charge around the (single) flavin binding site (10). Initial ATP-Mg\(^{2+}\) binding would overcome this repulsion and allow the subsequent FMN binding. However, our results invalidate this hypothesis showing that, in the absence of ATP-Mg\(^{2+}\), FMN preferably binds to the C-terminal domain in the absence of adenine nucleotides (Tables 1, 2 and 3). Saturation of \(\Delta(1-182)\)FADS with ADP-Mg\(^{2+}\) considerably enhanced the affinity of this domain for RF and, especially, for FMN (Figure 9). The positive cooperativity of ADP-Mg\(^{2+}\) on the binding of RF to \(\Delta(1-182)\)FADS is reciprocal (28). Therefore, when any of the two ligands is bound to \(\Delta(1-182)\)FADS, there is a 9-fold increase in the binding affinity of the second one. Similarly, ADP-Mg\(^{2+}\) enhances the CaFADS ability to bind RF and promotes the appearance of a second FMN binding site (Table 2), that of FMN as a product of the phosphorylation reaction. The fact that FMN only binds to the C-terminal domain in the presence of the second reaction product, ADP, agrees with ADP leaving the phosphorylation site prior to FMN (10,16,27). At the highest [Mg\(^{2+}\)] assayed, the affinity for FMN at both binding sites differs by 20-fold (Table 2). Comparison of these \(K_d\) values with that obtained in the simultaneous excess of ADP and FAD suggests that the highest affinity site must be the one at the N-terminal domain. Thus, ADP allows FMN to bind in the C-terminal region, while the affinity in the N-terminal site considerably increases. Therefore, positive cooperativity is also observed in the N-terminal region: when any of the two ligands is bound, the binding affinity of the other one increases 29-fold.

Therefore, ADP, Mg\(^{2+}\) and particularly ADP-Mg\(^{2+}\) produce a synergistic stabilising effect in the formation of the FADS:flavin:nucleotide complex. Although the complexes analysed represent combinations of products or substrate analogues that will never react with each other, similar effects might be expected for the reacting substrates. The positive cooperative binding effect could be consequence of ADP-Mg\(^{2+}\) mediating additional interactions between the protein and the flavin, as indicated by the favourable enthalpic contribution for the simultaneous binding of RF and ADP-Mg\(^{2+}\) (Figure 9). On the other hand, binding of one of the ligands could induce a conformational change that would allow the tighter binding or better accommodation of the second one in the active site. Both possibilities are not mutually exclusive.

In an attempt to dissect the binding phenomena, the relative enthalpic (\(\Delta H\)), entropic (\(\Delta S\)) and free energy changes (\(\Delta G\)) associated with the binding of flavins to the binary complex FADS:ADP versus that to free FADS have been calculated, when possible, according to \(\Delta G = \Delta G_{binary} - \Delta G_{free}\), \(\Delta H = \Delta H_{binary} - \Delta H_{free}\) and \(-T\Delta S = -T\Delta S_{binary} + -T\Delta S_{free}\) (binary and free refer to the binding of a flavin to the FADS:ADP complex and free FADS, respectively). ADP-Mg\(^{2+}\) favoured the binding of RF to \(\Delta(1-182)\)FADS by -1.3 kcal/mol. This relates with a much more favourable enthalpic contribution, -9.9 kcal/mol, indicating that ADP-Mg\(^{2+}\) promotes the appearance of new favourable polar interactions between \(\Delta(1-182)\)FADS and RF. The effect of ADP-Mg\(^{2+}\) on the interaction of FMN with \(\Delta(1-182)\)FADS, or with the C-terminal of FADS, is even more drastic, since flavin binding only occurs when ADP and Mg\(^{2+}\) are simultaneously bound. Moreover, the presence of ADP-Mg\(^{2+}\) promotes not only a favourable enthalpic contribution,
but also a favourable entropic one, suggesting cooperation of ADP-Mg\(^{2+}\) in the preformation of the FMN binding site (Figures 8 and 9).

When dissecting the thermodynamics of FMN binding to the N-terminal domain saturated with ADP-Mg\(^{2+}\) versus the free domain, the enhanced interaction (\(\Delta\Delta G = -2.0\) kcal/mol) correlated with a considerably unfavourable decrease in the enthalpic contribution and a considerably favourable increase in the entropic one (\(\Delta\Delta H = +18.8\) kcal/mol and \(-T\Delta\Delta S = -20.8\) kcal/mol). The presence of ADP-Mg\(^{2+}\) appears to cause a favourable hydrophobic effect for the FMN interaction that was not observed in its absence, and the adenine nucleotide binding “freezes” a protein conformation that favours flavin binding. While Mg\(^{2+}\) reduces both the enthalpic and entropic contributions in the interaction with substrates, nucleotide binding free energy is employed in reducing the conformational energy penalty occurring in flavin binding. This is in agreement with kinetic analyses suggesting that during the adenylylation cycle the adenine nucleotide binds prior to FMN (10). Despite the observed cooperativity, all substrates bind in the absence of the other one. Therefore, FADS appears to show a cooperativity in substrate binding less strict than that reported in other enzymes (29).

In conclusion, CaFADS holds two independent adenine nucleotide sites and two independent flavin binding sites, which correlate with the two different reactions catalysed. Substrates modulate the structural organisation of the enzyme’s catalytic sites, and the affinity for the rest of the ligands. Additionally, the energetics of the interaction, and the protein function itself, are related to the structural dynamics of the protein. The lack of some of the loops putatively involved in substrate binding in the crystal structure of TmFADS, together with the in silico models derived for the CaFADS, clearly suggest a high flexibility for these particular loops (13-15). The structural data reported for monofunctional NTs and RFKs indicate drastic conformational changes associated with nucleotide and flavin binding, by promoting strong interactions and modifying the coordination between the active site residues and the Mg\(^{2+}\), ATP and flavin substrates, while simultaneously shielding the active site from the solvent (16,17,27,30-34). Therefore, it is feasible to expect that during its catalytic cycle FADS will undergo a series of conformational rearrangements in order to optimally allocate substrates at the active site and to stabilise transition-states (34,35).

REFERENCES


**FOOTNOTES**

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Abbreviations: FADS, FAD Synthetase; LF, lumiflavin; RF, riboflavin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; RFK, riboflavin kinase; NT, nucleotydyltransferase; ADP, adenosine di-phosphate; AMPPNP, 5′-adenosine 5′-(β,γ-imido) tri-phosphate; ATP, adenosine tri-phosphate; PPi, pyrophosphate; DSC, differential scanning calorimetry; ITC, isothermal titration calorimetry.

**FIGURE LEGENDS**

Figure 1. (A) Scheme of the reactions catalysed by FADS. (B) Three-dimensional model for the CaFADS showing the putative binding sites for its different ligands (shown in sticks, Mg2+ shown as spheres) (15).
**Figure 2.** Selected spectroscopic properties of CaFADS. (A) Circular dichroism spectra (molar ellipticity by residue) of FADS in the far-UV and near-UV (inset) regions recorded at 25 °C in 5 mM and 20 mM sodium phosphate, pH 7.0, respectively. Vis difference spectra obtained upon titration of FADS with RF (solid line), FMN (dotted line) and FAD (dashed line) in the absence (B) and presence (C) of 10 mM MgCl2 and 1 mM AMPPNP. Spectra were recorded in 50 mM Tris/HCl, pH 8.0 at 25 °C. (D) Difference spectroscopy titration curves for the interaction of FADS with RF (closed squares), FMN (crosses) and FAD (open circles).

**Figure 3.** Raw data thermograms (upper panels) and binding isotherms with integrated heats (lower panel) for the titration of (A) FADS (11.4 μM in the calorimetric cell) with ATP (512 μM in the syringe), (B) FADS (14.0 μM) with ADP (541 μM), and (C) FADS (12.0 μM) with AMPPNP (542 μM) in 20 mM sodium phosphate, pH 7.0, 10 mM MgCl2 at 25°C.

**Figure 4.** Thermodynamic dissection of the interaction of FADS with the adenine nucleotides ATP, ADP and AMPPNP at different MgCl2 concentrations. The binding Gibbs energy (ΔG), enthalpy (ΔH) and entropy (-TΔS) are represented in striped, dark grey and light grey bars, respectively. Any negative value represents a favourable contribution to the binding, whereas a positive value represents an unfavourable contribution. Mg2+ concentrations and binding stoichiometry (N) are indicated in the figure.

**Figure 5.** Raw data thermograms (upper panels) and binding isotherms with integrated heats (lower panel) for the titration of (A) FADS (11.0 μM in the calorimetric cell) with FMN (297 μM in the syringe), (B) FADS (12.3 μM) with FAD (305 μM) in the presence of 10 mM Mg2+, and (C) FADS (5.0 μM) with RF (152 μM) in 20 mM sodium phosphate, pH 7.0, 25°C.

**Figure 6.** Thermodynamic dissection of the interaction between FADS with FAD, FMN, RF and LF. Other conditions as in Figure 4.

**Figure 7.** Raw data thermograms (upper panels) and binding isotherms with integrated heats (lower panel) for the characterisation of the ternary interactions including FADS, ADP and the flavin nucleotides in 20 mM sodium phosphate pH 7.0, 25°C. (A) FADS (17.0 μM in the calorimetric cell) was titrated with FMN (295 μM in the syringe) in the presence of 0.5 mM ADP and 10 mM Mg2+, (B) FADS (15.2 μM) was titrated with FAD (276 μM) in the presence of 0.5 mM ADP and 10 mM Mg2+, and (C) FADS (3.7 μM) was titrated with RF (174 μM) in the presence of 0.45 mM ADP and 0.8 mM Mg2+.

**Figure 8.** Thermodynamic dissection of the interaction of the FADS:ADP preformed complex with FAD, FMN, RF and LF. Other conditions as in Figure 4.

**Figure 9.** Left: Raw data thermograms (upper panel and inset) and binding isotherms with integrated heats (lower panel) for the interaction of Δ(1-182)FADS (19 μM in the calorimetric cell) and FMN (308 μM in the syringe) in 20 mM sodium phosphate pH 7.0, 10 mM Mg2+ at 25°C, both in the absence (inset in the upper panel and open squares in the lower panel) and in the presence of ADP (upper panel and closed squares in the lowest panel). Right: Thermodynamic dissection of the interaction between the Δ(1-182)FADS and its ligands. The thermodynamic parameters for the interaction of the enzyme-ADP preformed complex with RF and FMN are also shown. Other conditions as in Figure 4.
### Table 1: Stoichiometry and interaction parameters for the FADS:nucleotide and FADS:flavin interaction determined by ITC. Data obtained at 25°C in 20 mM sodium phosphate, pH 7.0.

<table>
<thead>
<tr>
<th>FADS Ligand</th>
<th>N</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.8 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
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<tr>
<td>ATP</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>ADP</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>AMPPNP</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>FAD</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>FMN</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>RF</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>LF</td>
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### Table 2: Stoichiometry and interaction parameters for ligand binding to FADS in the presence of a second ligand determined by ITC. Data obtained at 25°C in 20 mM sodium phosphate, pH 7.0.

<table>
<thead>
<tr>
<th>Titrating Ligand</th>
<th>Saturating Ligand</th>
<th>N</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (μM)</th>
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<tr>
<td></td>
<td></td>
<td>0 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.8 mM Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
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<tr>
<td>LF</td>
<td>ADP, FAD</td>
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<td>ADP</td>
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<tr>
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<sup>a</sup> Most likely value based on the analysis of the enthalpy and entropy contributions.

<sup>b</sup> Very weak interaction is observed.
Table 3: Stoichiometry and interaction parameters for the (Δ1-182)FADS:ligand interaction determined by ITC. Data obtained at 25°C in 20 mM sodium phosphate, pH 7.0.

<table>
<thead>
<tr>
<th>Titrating Ligand</th>
<th>Saturating Ligand</th>
<th>N</th>
<th>K_d (μM)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td>0 mM Mg^{2+}</td>
<td>10 mM Mg^{2+}</td>
</tr>
<tr>
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<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ADP</td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>FAD</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FMN</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
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<tr>
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<td>1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*a* No interaction is observed under these conditions.

*b* Very weak interaction is observed.
Figure 2

A

B

C

D

\[ \text{Wavelength (nm)} \]

\[ \text{Wavelength (nm)} \]

\[ \text{[Flavin] (\mu M)} \]

\[ \Delta e (\mu M^{-1} \text{cm}^{-1}) \]

\[ \Delta \text{Abs} \]
Figure 3

Graph A: 
- dQ/dt (μcal/s) vs. [ATP]_r/[FADS]_r
- Time (min) from 0 to 210
- Q (kcal/mol of injectant) from 0 to -0.8

Graph B: 
- dQ/dt (μcal/s) vs. [ADP]_r/[FADS]_r
- Time (min) from 0 to 210
- Q (kcal/mol of injectant) from 0 to -2

Graph C: 
- dQ/dt (μcal/s) vs. [AMPPNP]_r/[FADS]_r
- Time (min) from 0 to 210
- Q (kcal/mol of injectant) from 0 to -6
Figure 4

[Graph showing energy changes in Kcal/mol for ATP, ADP, and AMPPNP at different Mg^2+ concentrations (0 mM, 0.8 mM, 10 mM).]
Figure 5
Figure 7

(A) 

(B) 

(C) 

Q (kcal/mol of injectant) vs. [FMN]/[FADS]$_T$

Q (kcal/mol of injectant) vs. [FAD]/[FADS]$_T$

Q (kcal/mol of injectant) vs. [RF]/[FADS]$_T$
Figure 9

[Graphs showing the relationship between [FMN]/[FADS]_r and time (min), dQ/dt (μcal/s), Q (kcal/mol of injectant), and Kcal/mol for different Mg^2+ concentrations (0 mM, 10 mM) and substrates (ATP, ADP, RF, FMN).]

0 mM Mg^2+ 10 mM Mg^2+ 10 mM Mg^2+ + ADP

N=1 N=1 N=1

ATP

ADP

RF

FMN

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