Phosphorylation of Bovine Adrenodoxin by Protein Kinase CK2 Affects the Interaction with Its Redox Partner Cytochrome $P450_{scc}$ (CYP11A1)†

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ABSTRACT: Adrenodoxin (Adx), a [2Fe-2S] vertebrate-type ferredoxin, transfers electrons from the NADPH-dependent flavoprotein Adx reductase (AdR) to mitochondrial cytochrome P450 enzymes of the CYP11A and CYP11B families, which catalyze key reactions in steroid hormone biosynthesis. Adx is a known phosphoprotein, but the kinases that phosphorylate Adx have remained mostly obscure. The aim of this study was to identify previously unknown Adx phosphorylating kinases and to acquire a deeper insight into the functional consequences of such a modification. Here, we show for the first time that bovine Adx is a substrate of protein kinase CK2, whereas bovine CYP11A1, CYP11B1, and AdR are not phosphorylated by this kinase. CK2 phosphorylation of mature Adx requires the presence of both the catalytic α-subunit and the regulatory *β*-subunit of CK2 and takes place exclusively at residue Thr-71, which is located within the redox partner interaction domain of the protein. We created two Adx mutants, Adx-T71E (imitating a phosphorylation) and Adx-T71V (which cannot be phosphorylated at this site), respectively, and investigated how these mutations affected the interaction of Adx with its redox partners. These data were supplemented with detailed spectroscopic and functional assays using the phosphorylated protein. All Adx species behaved like wild type (Adx-WT) with respect to their redox potential, iron−sulfur cluster symmetry, and overall backbone structure. Substrate conversion assays catalyzed by CYP11A1 showed an increase in product formation when Adx-T71E or CK2-phosphorylated Adx were used as electron carrier instead of Adx-WT, whereas the activity toward CYP11B1 was not altered using these Adx species. Additionally, Adx-T71E represents the only full-length Adx mutant which leads to an increase in CYP11A1 product formation. Therefore, characterizing this full-length mutant helps to improve our knowledge on the functional effects of phosphorylations on complex redox systems.

Ferredoxins comprise a family of iron−sulfur proteins that are widely distributed in bacteria, yeast, plants, and animals, where they participate in a broad variety of electron-transfer reactions. [2Fe-2S]-type ferredoxins are low molecular mass proteins (6−25 kDa) that are negatively charged at neutral pH and contain iron−sulfur clusters as a redox active group. Adrenodoxin (Adx)[1] is a well-studied representative of vertebrate-type [2Fe-2S] ferredoxins that carries electrons from the NADPH-dependent flavoprotein Adx reductase (AdR) to mitochondrial cytochrome P450 enzymes, for example, those of the CYP11 family, which catalyze key reactions in the biosynthesis of steroid hormones (*1, 2*). While phosphorylation of either AdR or mitochondrial P450s has not yet been reported, Adx is known to be phosphorylated by PKA (*3*−*5*). Nevertheless, a significant uncertainty exists about the identity of other Adx phosphorylating kinases and the relevant phosphorylation sites. In addition, the functional consequences of this phosphorylation is unclear, as phospho-Adx was reported to stimulate the activity of CYP11A1 and CYP11B1 (*4*) but to have no effect on cytochrome *c* reductase activity and even to inhibit renal steroid 1α-hydroxylase activity (*3, 5*). Moreover, studies were lacking on the investigation of phosphorylation-deficient Adx mutants. The aim of this work was to identify new Adx-phosphorylating kinases and to analyze the consequences of such a phosphorylation on the functionality of this iron−sulfur protein.

In general, covalent modifications of a protein with a charged group, such as a phosphate group, are likely to alter the steric, electrostatic, and hydrogen bonding properties of proteins. Phosphorylation might also significantly alter the redox properties of electron-transfer (ET) proteins by affecting the binding behavior toward its interaction partners. Previous studies have shown that the binding constants between Adx and its redox partners (AdR and CYP11A1) are significantly decreased by high ionic strength, underlining the importance of electrostatic interactions for complex formation between these proteins (*6*). Taking this into

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[1] Abbreviations: Adx, adrenodoxin; AdR, adrenodoxin reductase; PKA, cAMP-dependent protein kinase; StAR, steroidogenic acute regulatory protein; DMEM, Dulbecco's modified Eagle's medium; CD, circular dichroism.

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account, a phosphorylation of Adx, especially in the interaction domain (which comprises residues 67−87), is very likely to affect the functionality of this iron−sulfur protein. A comparison of kinase epitopes revealed two interesting phosphorylation motifs in this domain, a known cAMP-dependent protein kinase (PKA) motif at position Ser-88 (*4*) and a putative protein kinase CK2 motif at position Thr-71. Previous studies have suggested that bovine Adx is phosphorylated by PKA at position Ser-88 but is not phosphorylated by CK2 (*4*). However, these results are contradicted by recent studies which indicate that PKA does not phosphorylate Adx at position Ser-88 (Maucourt and Bernhardt, unpublished results). It was therefore our interest to investigate whether Adx is a substrate for CK2.

In this work, we report for the first time that Adx is efficiently phosphorylated by protein kinase CK2. By contrast, we found that this kinase does not phosphorylate other components of this electron-transport chain, bovine AdR, CYP11A1, and CYP11B1. Protein kinase CK2 is a highly conserved serine/threonine kinase which is found in many organisms and tissues and has been described as a pleiotropic, ubiquitous, and constitutive active protein kinase involved in cell cycle regulation, apoptosis, and cancer (*7*−*10*). The holoenzyme is a tetramer containing two α or two α′ subunits (or one of each) and two β subunits. The α and α′ subunits possess catalytic activity, whereas the β subunits, which are in vitro autophosphorylated by the holoenzyme, have regulatory functions. So far, more than 300 CK2 substrates have been identified, a finding that reflects the very simple phosphorylation motif required by this enzyme (Thr, Ser-$(X)_{1-2}$-Glu/Asp). We demonstrate that CK2 phosphorylation occurs at residue Thr-71 of mature Adx. Moreover, using CK2-phosphorylated Adx as well as an Adx mutant that mimics phosphorylation at residue T71, Adx-T71E, we were able to show that this phosphorylation leads to a significant increase in the formation of pregnenolone from cholesterol, the reaction that is generally considered to be the rate-limiting step in steroid biosynthesis (*4*), whereas CYP11B1 product patterns were unchanged compared to Adx-WT. The findings presented in this work will help to increase our knowledge on the consequences of phosphorylations on the function of ferredoxins.

EXPERIMENTAL PROCEDURES

Chemicals. Radioactive compounds were obtained from Amersham Pharmacia Biotech (Freiburg, Germany) or NEN (Boston, MA); nonradioactive steroids were purchased from Sigma (Deisenhofen, Germany).

Mutagenesis, Bacterial Protein Expression, and Enzyme Purification. Nucleotide substitutions were performed using the QuikChange site-directed mutagenesis kit (Stratagene; La Jolla, CA) and oligonucleotides with the appropriate mutations (BioTez; Berlin, Germany). The introduction of the desired mutations was verified by automatic sequencing with a LiCor sequencer (MWG-Biotech; Ebersberg, Germany). Isolation of DNA fragments, plasmid preparations, ligations, and transformations of *E. coli* were conducted according to standard techniques. For bacterial expression, mutated Adx cDNAs were cloned into pET3d. The mutated Adx species were produced using *E. coli* BL21 (DE3) pLysE transformed either with plasmids containing the cDNA of WT-Adx (*11*, *12*) or with its derivatives that contain the Adx mutants as a host strain. Recombinant Adx and AdR were purified as described (*11*, *13*). Protein concentrations were calculated using $\epsilon_{414} = 9.8$ (mM * cm)$^{-1}$ for Adx (*14*) and $\epsilon_{450} = 11.3$ (mM * cm)$^{-1}$ for AdR (*15*). The different Adx species were expressed as holoproteins in the cytoplasm of *E. coli*. A typical expression yield was in the range of 16−18 mg * L^{-1} of highly purified protein. The final holoprotein index (A_{414}/A_{276}) was in the range of 0.85−0.9 for all purified Adx species. Isolation of CYP11A1 and CYP11B1 from bovine adrenal glands was performed as previously described (*16*, *17*). CYP11A1 and CYP11B1 concentrations were estimated by carbon monoxide difference spectra using $\epsilon_{450-490} = 91$ (mM * cm)$^{-1}$. Protein kinase CK2 holoenzyme and the catalytic (α) subunit were expressed and purified as described previously (*18*). The subunit composition of the recombinant CK2 holoenzyme used in these studies was $2\alpha 2\beta$.

In Vitro Phosphorylation by Protein Kinase CK2. Unless otherwise noted, phosphorylation reactions were carried out at 37 °C in a total volume of 100 μL (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 14 μg protein substrate, 1 μg CK2-holoenzyme, and 50 μM ATP). Samples used for radioactive labeling of Adx additionally contained 2 μCi $^{33}P\gamma$ATP. For the time-dependent phosphorylation studies, the reaction time was varied between 0 and 180 min. All other phosphorylation reactions were carried out for 120 min. To the samples that were used for the subsequent autoradiographic detection of phosphorylated Adx 100 μL sample buffer, 65 mM Tris-HCl, pH 6.8, 0.01% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol, and 2% SDS were added. Proteins were separated by SDS−PAGE, blotted onto a nitrocellulose membrane, and visualized by exposure to autoradiographic imaging plates (BAS-2500, Fuji; Stamford, CT) for 6 h. The obtained results were analyzed using the software TINA20. In vitro phosphorylation reactions performed for the subsequent functional or biophysical analysis of phosphorylated Adx contained 50 μM Adx (216 μg Adx), 15 μg CK2, and 250 μM ATP in CK2 reaction buffer (total volume: 300 μL). These samples were incubated for 2 h and afterward diluted in the buffer required for each experiment, respectively. Prior to using these samples, the degree of phosphorylation was determined with a luciferase assay as indicated below.

Luciferase Assays. CK2 activity and the degree of phosphorylated Adx was measured by quantifying the amount of ATP remaining in the solution after the kinase reaction had taken place. ATP consumption was determined with the Kinase-Glo luminescent kit (V6711, Promega; Madison, WI) according to the instructions of the manufacturer. Kinase reactions used for the determination of the degree of phosphorylation either contained 14 μg Adx, 1 μg CK2-holoenzyme, and 50 μM ATP or 216 μg Adx, 15 μg CK2, and 250 μM ATP. Samples created for the kinetic analysis of the phosphorylation of Adx by CK2 always consisted of 1 μg CK2-holoenzyme, 50 μM ATP, as well as different Adx concentrations (0−40 μM). The incubation time of these reactions was 30 min. After the phosphorylation reaction had taken place, the samples were 25-fold (samples with 50 μM ATP) or 125-fold (samples containing 250 μM ATP) diluted in PBS. One hundred microliters of the diluted sample was then loaded onto a solid white 96 well plate (Nunc;

Wiesbaden, Germany) and supplemented with 100 μL luminescent reagent. After 10-min incubation at room temperature, the luminescent signal was recorded on a Genios reader (TECAN; Groedig, Austria). Instrument settings were as follows: integration time was 150 ms and the signal gain was 100.

Spectroscopic Methods. Absorption spectra in the UV−vis region were recorded at room temperature on a double-beam spectrophotometer UV2101PC (Shimadzu; Kyoto, Japan). Reduction of Adx was accomplished by adding sodium dithionite up to a concentration of 150 μM in an anaerobic atmosphere. CD spectra of oxidized Adx species in the range of 250−650 nm were recorded on a Jasco 715 spectropolarimeter as described previously (*19*). All Adx samples were diluted in 50 mM HEPES buffer containing 2 mM DTE. Possible changes in the secondary structures of the different Adx species were investigated by recording CD spectra in the range of 195−260 nm. These experiments were carried out in 0.1-cm quartz cuvettes containing 10 μM Adx. EPR measurements were performed on a Bruker ESP300E as described previously (*20*). Standard measurement parameters were modulation frequency, 100 kHz; microwave strength, 10 mW; modulation amplitude, 10 G; and time constant, 0.3 s.

Biophysical and Biochemical Parameters of the Adrenodoxin Mutants. Redox potentials were measured using the dye photoreduction method with Safranin T as indicator and mediator (*21*). Data were determined from at least three independent experiments and analyzed according to the Nernst equation. Molar extinction coefficients of the Adx mutants were derived by determining the total amount of protein with a BCA assay (Pierce; Rockford, IL) as well as the absorption at 414 nm. The purity index of each Adx species was used to determine the exact amount of adrenodoxin in each sample. Calculation of the coefficients was done using the Lambert−Beer equation.

Cytochrome P450 Dependent Substrate Conversion. The CYP11A1 catalyzed conversion of cholesterol to pregnenolone was investigated in a reconstituted system according to the procedure of Sugano et al. (*22*) with slight modifications. Assays were performed at 37 °C for 10 min in 50 mM HEPES buffer (pH 7.4) with 0.05% Tween 20. Each sample contained 0.5 μM AdR, Adx-WT, or Adx mutants in varying concentrations (0.2−4.0 μM), 0.4 μM CYP11A1, 400 μM cholesterol, and a NADPH regenerating system consisting of $MgCl_2$ (1 μM), glucose-6-phosphate (5 μM), and glucose-6-phosphate dehydrogenase (1 U). Substrate conversion was started by addition of NADPH (100 μM). After the reaction, steroids were converted into their corresponding 3-one-4-en forms by cholesterol oxidase and extracted with chloroform. The extracted steroids were then separated on a Jasco reversed-phase HPLC system of the LC800 series using a 3.9 × 150 mm Waters Nova-Pak C_{18} column at 45 °C. The mobile phase used for the separation was a mixture of acetonitrile/2-propanol (30:1). Samples created for the analysis of the CYP11B1 catalyzed conversion of deoxycorticosterone to corticosterone contained 0.5 μM AdR, varying concentrations of Adx-WT or Adx mutants (0.2−40.0 μM), 0.4 μM CYP11B1, 400 μM desoxycorticosterone, and the above-mentioned NADPH regenerating system. The sample buffer as well as the reaction conditions were as described above. After completion of the reaction, steroids were directly extracted via chloroform, resuspended in acetonitrile, and loaded onto a Waters Nova-Pak C_{18} column kept at 25 °C. Steroid separation was achieved with an isocratic solvent system of acetonitrile/water (60:40). In both cases, product quantification was performed by correlating the product peak integrals with the peak area of a known internal standard (5 nmol cortisol) which was added prior to the chloroform extraction. K_m and V_{max} values were determined by plotting the substrate conversion velocity versus Adx concentration and applying Michaelis−Menten kinetics (hyperbolic fit). The velocity of the Adx dependent product formations was expressed in nmol product * min^{-1} * nmol $CYP11A1^{-1}$ or in nmol product * min^{-1} * nmol $CYP11B1^{-1}$, respectively. Substrate conversion assays using phosphorylated Adx as electron carrier were performed with a single ferredoxin concentration. This concentration corresponds to the amount of Adx needed to achieve maximal substrate conversion as determined in experiments with the Adx-WT and Adx-T71 mutants. In CYP11A1-assays, the applied concentration of phosphorylated Adx was 3 μM, while for CYP11B1 reactions it was 10 μM. All assays were repeated at least four times.

Cell Culture, Transient Transfection, and Enzymatic Assays. COS-1 cells were grown at 37 °C and 6% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 0.1 mg * mL^{-1} streptomycin, 100 U * mL^{-1} penicillin, 1 mM pyruvate, and 4 mM L-glutamine. Transfections were performed using Effectene Transfection Reagent (Qiagen; Hilden, Germany) according to instructions of the manufacturer. To assay for CYP11A1 activity, cells were cotransfected with pSVL-bCYP11A1 (*23*) and Adx-expression plasmids as indicated. After transfection, cells were incubated with 1 μM 22R-hydroxycholesterol and 66.7 nCi * mL^{-1} [^{14}C] 22R-hydroxycholesterol for 24 h. Cell culture supernatants were extracted with chloroform and the organic phase was dried under vacuum. After resuspension of the extracted steroids in 10 μL chloroform, the samples were spotted onto glass-backed silica-coated HPTLC plates (Kieselgel 60 F_{254}, Merck; Darmstadt, Germany). In addition, small amounts of non-radioactive steroids were used as references. HPTLC plates were developed twice in diethyl ether/*n*-hexane/acetic acid (45:45:3, v/v/v), and steroids were identified after exposure to Fuji imaging plates. Steroid quantification was performed on a phosphoimager (BAS-2500, Fuji; Stamford, CT). To assay for CYP11B1 activity, cells were cotransfected with pSVL-bCYP11B1 (*23*) and Adx-expression plasmids as indicated. After transfection, cells were incubated for 48 h either with 10 μM 11-deoxycortisol and 167 nCi * mL^{-1} [^{3}H]11-deoxycortisol or with 10 μM 11-deoxycorticosterone and 8.3 nCi * mL^{-1} [^{14}C]11-deoxycorticosterone, respectively. As described above, cell culture supernatants were extracted with chloroform and analyzed by HPTLC. In this case, HPTLC plates were developed twice in chloroform/methanol/water (300:20:1, v/v/ v). Identification and quantification of the separated steroids was performed as described above. All data presented in this work were calculated from three independent experiments.

Structure Homology Modeling of Adrenodoxin T71 Mutants. All computer models were created by mutating residue Thr-71 of bovine Adx (1AYF.pdb) to valine or glutamic acid, respectively, with the program SYBYL 6.8. The obtained

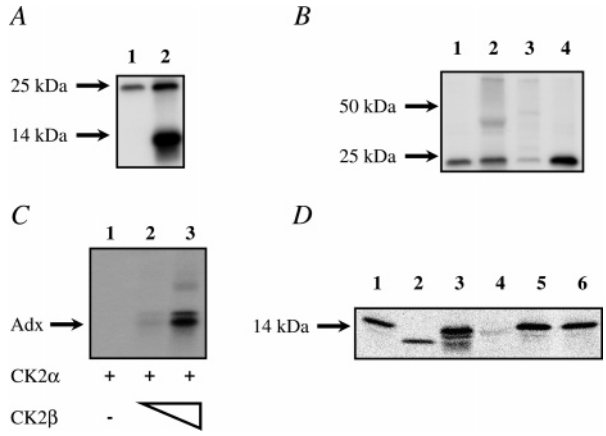

FIGURE 1: In vitro phosphorylation experiments. Radioactive kinase assays, SDS−PAGE, and autoradiography were carried out as described in Experimental Procedures. After autoradiography, Western blot analysis was carried out to confirm the identification of the protein bands. All results shown are representative for six independent experiments. (A) Phosphorylation of mature bovine Adx-WT (M_r = 14.0 kDa) by protein kinase CK2 holoenzyme. As expected, autophosphorylated CK2β is detectable at 25 kDa. Lane 1, control; lane 2, Adx-WT. (B) Mature bovine AdR (M_r = 50.3 kDa), mature CYP11A1 (M_r = 56.4 kDa), and mature CYP11B1 (M_r = 55.1 kDa) are not phosphorylated by CK2 under the same conditions. Lane 1, control; lane 2, AdR; lane 3, CYP11A1; lane 4, CYP11B1. (C) Phosphorylation of Adx by CK2α requires the presence of CK2β. Kinase assays were performed as above using Adx-WT as phosphorylation substrate and the catalytic CK2α subunit either without (lane 1) or with increasing concentrations of the regulatory CK2β subunit (lanes 2 and 3). CK2α alone does not phosphorylate Adx-WT; the formation of phospho-Adx increases with growing concentrations of CK2β. (D) Phosphorylation of Adx mutants lacking putative CK2 phosphorylation sites. Kinase assays were performed as above using CK2 holoenzyme and Adx mutants as indicated. Putative CK2 phosphorylation sites in bovine Adx are Ser-1, Ser-2, Ser-28, Thr-71, and Ser-125. All Adx mutant species tested were readily phosphorylated except for Adx-T71V. Lane 1, Adx-WT; lane 2, Adx^{4-108}; lane 3, Adx^{4-128}; lane 4, Adx-T71V; lane 5, Adx-S125A; lane 6, Adx-S28A.

structures were solvatized with XFIT (1000 water molecules) followed by 300 steps of energy minimization using the conjugate gradients algorithm and the Tripos force field (SYBYL 6.8, Tripos; St. Louis, MO). The template used for the models is a crystal structure of a truncated oxidized bovine adrenodoxin that consists of amino acids 4−108 of the mature protein (*24*).

RESULTS

Bovine Adx, but Not Bovine AdR, CYP11A1, or CYP11B1, Are Phosphorylated in Vitro by Protein Kinase CK2. Bioinformatic analysis using the prosite database (http://www.expasy.ch/prosite/) revealed the presence of several putative CK2 phosphorylation sites in bovine AdR, Adx, CYP11A1, and CYP11B1. However, the significance of such findings is at best moderate, since this very abundant motif only requires the presence of an acidic side chain three residues downstream of a serine or threonine residue. To test whether these proteins are indeed substrates of CK2, we performed in vitro kinase assays using either bacterially expressed recombinant bovine proteins (AdR, Adx) or proteins purified from bovine adrenals (CYP11A1, CYP11B1). We found that Adx was phosphorylated by CK2 (Figure 1A), while AdR, CYP11A1, and CYP11B1 were not (Figure 1B). Further experiments showed that the human (hAdx; (*25*)) and fission yeast (etp1^{fd}; (*26*)) homologues of bovine Adx could also be in vitro phosphorylated by protein kinase CK2 (data not shown), indicating a potential evolutionary conservation of this phosphorylation event.

Phosphorylation of Adrenodoxin Depends on the Presence of Both CK2 Subunits. To determine whether Adx is also phosphorylated by just the catalytic CKα subunit, Adx was incubated with CK2α and $^{32}P\gamma$ATP in the absence or presence of increasing concentrations of the CK2β-subunit. Phosphorylated proteins were analyzed on a SDS-polyacrylamide gel and subjected to autoradiography. As shown in Figure 1C, no Adx phosphorylation was detectable in the absence of CK2β, while the presence of increasing amounts of this regulatory subunit leads to an increasing signal. This experiment indicates that phosphorylation of Adx requires the presence of the CK2 holoenzyme.

Threonine-71 is the Major CK2 Phosphorylation Site in Adx. The interaction between WT-Adx and CK2 was first characterized using luciferase assays. This type of assay represents a sensitive way to determine the amount of ATP present in a solution by measuring the bioluminescence intensity. The emitted luminiscence signal is correlated with the amount of ATP present in the sample and is therefore inversely correlated with kinase activity.

The V_{max} and K_m values for the phosphorylation of Adx by CK2 can be extracted by keeping the reaction time and concentrations constant for each reaction component except for Adx and subsequently quantifying ATP production. Determination of the ATP concentration in the sample was achieved by using an ATP-calibration curve (0−2 μM ATP). The obtained results from these experiments, assuming a single phosphorylation site in mature Adx, is shown in Figure 2A. The values of V_{max} and K_m, 0.0075 nmol Adx * min^{-1} * μg CK2^{-1} and 2.4 μM, respectively, fit in the range of data obtained using different peptides as CK2 substrates (*27*). From this kinetic data, we calculated the time required to completely phosphorylate 1 nmol Adx in 100 μL (10 μM Adx) to be approximately 120 min. This result was verified through applying the above-described reaction parameters in time-dependent phosphorylation reactions using $^{33}P\gamma$ATP. The Adx concentration chosen for these experiments is higher than the determined K_m value and should therefore enable a maximal turnover rate by CK2 assuming a single phosphorylation site in mature Adx. From this time-dependent experiment, the maximal amount of phosphorylated Adx was obtained after the estimated reaction time, which suggests the existence of a single CK2 phosphorylation site in Adx (Figure 2B).

Five putative CK2 phosphorylation motifs [**S/T**]-X-X-[D/E] (*28*) are present in the primary sequence of bovine Adx; they are all located in the mature form of the protein and their sequences are **S^1**-S-S-E^4, **S^2**-S-E-D^5, **S^{28}**-L-L-D^{31}, **T^{71}**-D-E-E^{74}, and **S^{125}**−K-I-E^{128}, respectively. To identify the exact phosphorylation site of CK2 in Adx, we conducted in vitro kinase assays using mature full-length Adx (Adx-WT) and two truncated mutants, Adx^{4-128} and Adx^{4-108}, both of which constitute active proteins (*19*) and are essentially folded like Adx-WT (*24*). All three Adx species were readily phosphorylated by the CK2 holoenzyme (Figure 1D), thus pointing to phosphorylations at Ser-28 or Thr-71. The additional two weak bands exhibited by Adx^{4-128} correspond to degraded Adx species. Since CK2 phosphorylation of

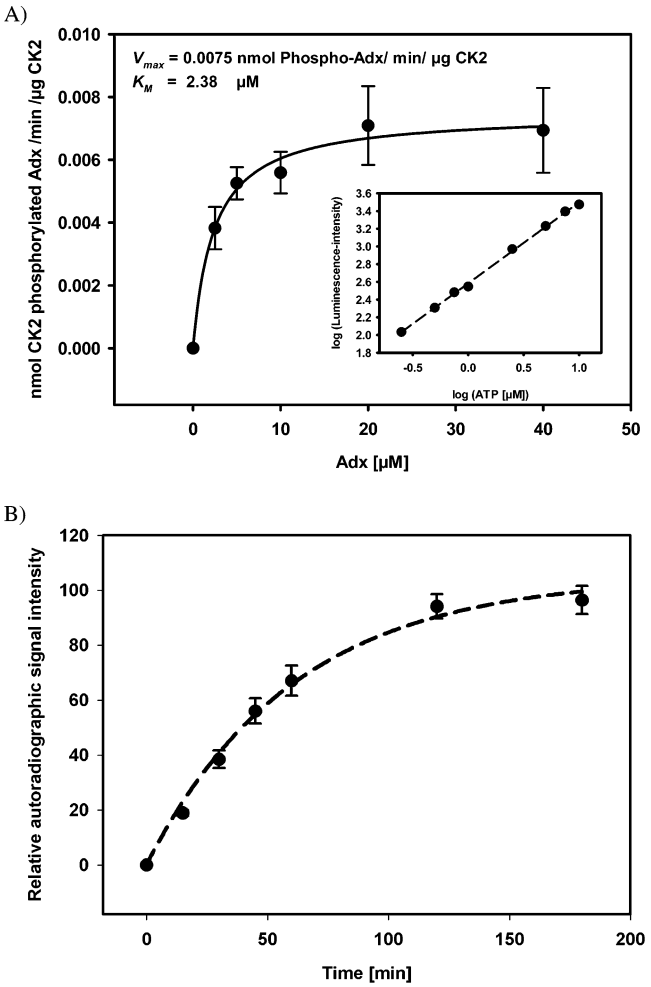

FIGURE 2: Kinetic analysis of the interaction between WT-Adx and CK2. (A) Determination of the ATP amount present after the kinase reaction has taken place was performed with the Kinase Glow kit from Promega, according to the manufacturer's instructions. Samples created for this purpose contained 1 μg CK2-holoenzyme and 50 μM ATP as well as different Adx concentrations (0−40 μM) in CK2 reaction buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT). The reaction time was set to 30 min. The insert shows an ATP concentration calibration curve used for the determination of ATP in the samples (standard deviation was ±5%) which is inversely correlated with the amount of phosphorylated Adx present. K_M and V_{max} values were determined by fitting the data hyperbolically. (B) Time-dependent phosphorylation of 1 nmol Adx by 1 μg CK2 using $^{33}P\gamma$ATP. After different incubation times (0−180 min), the reactions were stopped by addition of sample buffer. Proteins were then separated by SDS−PAGE, blotted onto a nitrocellulose membrane, and visualized by exposure to autoradiographic imaging plates for 6 h. Analysis was performed using the software TINA20. The autoradiographic signal intensity was then blotted against the incubation time. The maximal phosphorylation of Adx was accomplished after 120 min. The dashed line was added to visualize the time-dependent phosphorylation.

Adx^{4-108} was somewhat weaker than that of Adx^{4-128}, it was assumed that Ser-125 could be a secondary CK2 site. Therefore, three Adx mutants that carry point mutations hindering a phosphorylation of the respective residue (S28A, T71V, and S125A, respectively) were cloned, purified from recombinant bacteria, and used as substrates for CK2 in vitro phosphorylation. In this experiment, Adx-S28A (lane 6) and Adx-S125A (lane 5) were readily phosphorylated, while Adx-T71V (lane 4) was not. The very weak band visible in lane 4 is assumed to be due to inefficient and unspecific CK2 phosphorylation of other residues of Adx-T71V. This experiment proves that Thr-71 is the only relevant CK2 phosphorylation site in bovine Adx. The degree of phosphorylation of 1 nmol WT-Adx by 1 μg CK2 after an incubation time of 120 min and measured with the luciferase assay was estimated to be 85 ± 5.5%. Assays performed with the mutant T71V showed that the degree of phosphorylation of this mutant was always below 5%, underlining that this residue is the only relevant CK2 phosphorylation site in mature Adx (data not shown).

Spectroscopic Characterization of Adx-WT and Both Adx-T71 Mutants. Generally, the [2Fe-2S] cluster of ferredoxins can be sensitively characterized by circular dichroism (CD) spectroscopy (*29*) or absorption spectroscopy. In the oxidized state, adrenodoxin shows positive maxima at 320, 414, and 455 nm. To elucidate the properties of a CK2-phosphorylated Adx, we cloned an Adx-T71E mutant by site-directed mutagenesis, expressed it in *E. coli*, and purified it as described in Experimental Procedures. The substitution of Thr residues by Glu is a well-established technique intended to mimic a constitutively phosphorylated protein (*30*). Additionally, the handling of such a mutant compared to the phosphorylated protein is much easier because no precautions have to be taken to ensure that the phosphate group is not hydrolyzed during the experiments (e.g., by phosphatase reactions or temperature jumps during the purification procedure). The mutant Adx-T71V was used as a negative control since it simulates a permanent unphosphorylated state.

UV−vis spectroscopic analysis of both Thr-71 mutants in their oxidized (Figure 3A) and reduced (Figure 3B) states revealed no changes compared to the corresponding spectra of wild-type adrenodoxin. In addition, the molar extinction coefficients of the mutants were within the range of the standard deviation of the Adx-WT coefficient (data not shown). Adx displays the compact ($\alpha + \beta$) fold typical for [2Fe-2S] ferredoxins and is organized into a large core domain, which contains the iron−sulfur cluster, and an interaction (recognition) domain, which includes the residues that are involved in the interactions with its redox partners (*24*). Residues Asp-5 to Cys-55 and Gly-91 to Pro-108 belong to the core domain, while side chains His-56 to Leu-90 form a large hairpin that bears the key residues for recognition of AdR and CYP11A1 and comprises the two α-helices *E* and *F*. The interaction domain is more flexible than the core domain, and this conformational flexibility may be relevant for the interaction with AdR and the P450s. With Thr-71 being the first residue of the *F*-helix, modifications at this position could not be excluded to have an influence on the folding of the interaction domain of Adx. To test this possibility, CD measurements were carried out in the range from 195 to 260 nm (Figure 4A) as well as from 250 to 650 nm (Figure 4B). The results of these experiments demonstrate that both Adx-T71 mutants displayed no relevant alterations in comparison to Adx-WT, which clearly shows that the backbone structure (Figure 4A) as well as the immediate iron−sulfur cluster surrounding environment (Figure 4B) of the oxidized mutants has not been significantly changed as compared to Adx-WT. EPR measurements of all three Adx proteins displayed no changes, with all curve shapes as well as the two principal *g* factors of 1.933 and 2.024 being exactly identical (data not shown). Taken together, these data suggest that the overall structure of the Thr-71 mutations as

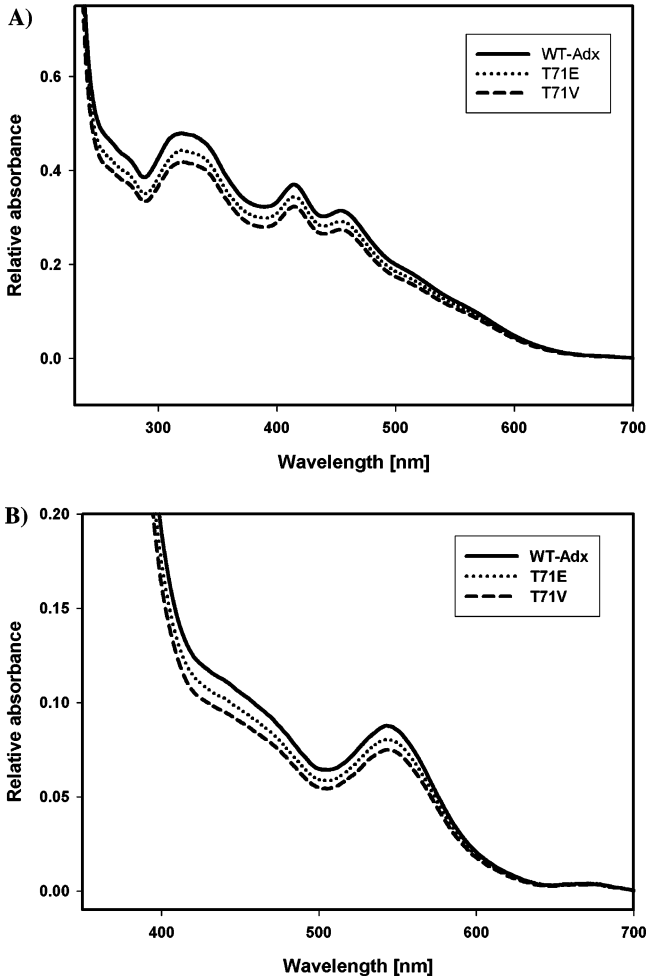

FIGURE 3: UV−vis spectra of oxidized (A) or reduced (B) Adx species. UV−vis spectra of different adrenodoxin species were recorded in 1-cm quartz cuvettes in the range from 250 to 700 nm (oxidized forms) or from 350 to 700 nm (reduced forms). Each sample contained 33 μM Adx in 50 mM HEPES buffer, pH 7.4. Reduction of the proteins was achieved in a glovebox by adding sodium dithionite up to a concentration of 150 μM. All curves were multiplied with a factor between 0.8 and 1.5 to obtain a better comparison between spectra.

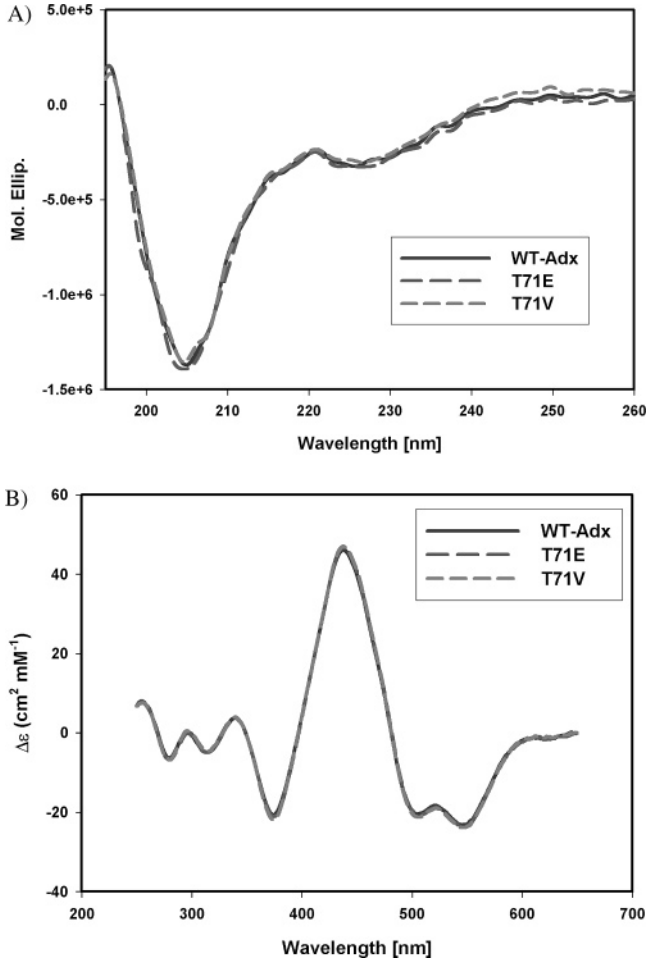

FIGURE 4: CD spectra of Adx-WT, Adx-T71V, and Adx-T71E. (A) CD spectra of Adx species in the range of 195−260 nm. Measurements were performed with 10 μM Adx in 5 mM HEPES buffer, pH 7.4, using a 0.1-cm quartz cuvette. (B) CD spectra of different oxidized Adx species in the range of 250−650 nm. Measurements were performed with 40 μM Adx in 50 mM HEPES buffer, pH 7.4, and 2 mM DTE using a 1-cm cuvette.

well as the symmetry of the reduced cluster remains unaltered. Both UV−vis spectra as well as CD measurements with CK2 phosphorylated Adx revealed no changes compared to the mutant and the WT protein (data not shown) indicating that no significant structural changes have been introduced in the phosphorylated protein and verifying that the mutant T71E resembles the phosphorylated protein.

Redox Properties of the Different Adx Species. The redox potential of an electron-transfer protein indicates its ability to accept and donate electrons, and changes in the redox potential of Adx induced by the substitution of a residue near the iron−sulfur cluster, Thr-71, could alter the functionality of this electron-transfer protein. Our experiments that probed the redox properties of these mutants showed that in all cases the iron−sulfur cluster was readily reduced by sodium dithionite like Adx-WT. The midpoint redox potentials for Adx-WT and mutant ferredoxins determined by spectrophotometric redox measurements (using Safranin T as indicator and mediator) are -269.2 ± 4.3 mV for Adx-WT, -272.1 ± 3.4 mV for Adx-T71V, and -270.1 ± 3.0 mV for Adx-T71E. The redox potential measured for CK2 phosphorylated Adx (-268.1 ± 4.5 mV) also revealed no relevant changes. Thus, CK2 phosphorylated Adx and both mutants display no significantly altered redox potentials compared to Adx-WT, which suggests that Thr-71 does not contribute to the modulation of the redox potential in Adx.

In Vitro Formation of Pregnenolone. In mammalian steroidogenesis, Adx transfers electrons from NADPH-reduced adrenodoxin reductase to mitochondrial cytochrome P450 enzymes. Two different bovine mitochondrial P450 isoenzymes have been characterized to date, CYP11A1 (*31*) and CYP11B1 (*32, 33*). The recognition between the different components is mainly based on electrostatic interactions (*6*). The acidic residues Asp-72, Glu-73, Asp-76, and Asp-79 of Adx play an essential role in the charge-driven molecular recognition mechanisms (*34*−*36*) by interacting with positively charged amino acids on the surface of AdR and CYP11A1, respectively (*37, 38*). Therefore, it is conceivable a priori that additional negative charges introduced by Thr-71 phosphorylation or substitution of Thr by Glu might enhance complex formation between Adx and its redox partners. The interaction of Adx with CYP11A1 promotes the binding of cholesterol, and optical difference spectroscopy experiments (*39*) reveal that this binding causes a shift of the low spin (417 nm) to the high spin (393 nm) form of the cytochrome heme iron. We determined a K_D value of

Table 1: Enzymatic Activity of Adx-T71E and Adx-T71V Compared to Adx-WT[a]

	CYP11A1 activity		CYP11B1 activity	
Adx species	V_{max} [nmol pregnenolone/min * nmol cytochrome P450]	K_m [μM]	V_{max} [nmol corticosterone/min * nmol cytochrome P450]	K_m [μM]
Adx-WT	3.82 ± 0.24	0.48 ± 0.09	4.40 ± 0.21	5.60 ± 0.44
Adx-T71E	5.32 ± 0.34	0.56 ± 0.18	4.20 ± 0.24	4.90 ± 0.76
Adx-T71V	3.85 ± 0.36	0.58 ± 0.19	3.70 ± 0.38	6.60 ± 0.62

[a] CYP11A1 dependent cholesterol conversion to pregnenolone was studied in the presence of AdR and either Adx-WT or Adx-T71 mutants by HPLC analysis. Data from at least four independent measurements were averaged, plotted against the corresponding Adx concentration, and fitted hyperbolically (Michalis−Menten kinetics).

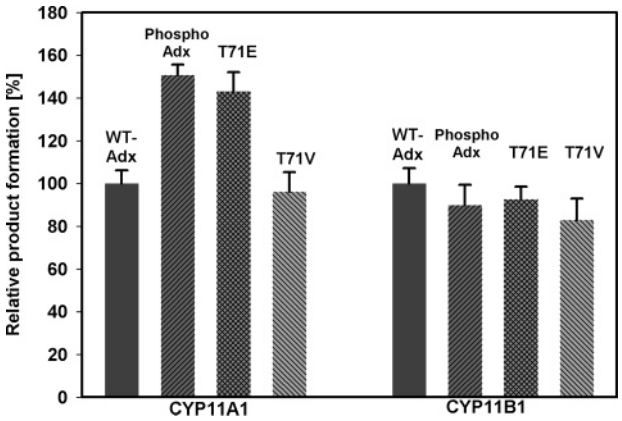

FIGURE 5: Comparison between the maximal product formation velocity by CYP11A1 and CYP11B1 using different Adx species. Relative values for phospho-Adx were determined from four independent measurements using the amount of Adx needed for maximal pregnenolone (3 μM) or corticosterone (10 μM) formation. Maximal substrate conversion of the Adx mutants was determined by concentration rows. Assays were performed with CYP11A1 or CYP11B1 as indicated. Product formation velocity using WT-Adx was set to 100% to compare the distinct substrate conversion efficiency by different Adx species.

3.6 μM for Adx-WT and a K_D value of 1.0 μM after CK2 phosphorylation of Adx (data not shown), which indicates that phosphorylation by CK2 increases the CYP11A1 binding affinity of Adx. To directly measure the effect of the Adx-T71 mutations on the substrate conversion of cholesterol to pregnenolone by CYP11A1 and the conversion of 11-desoxycorticosterone to deoxycorticosterone by CYP11B1, we performed in vitro steroid hydroxylation assays. In these experiments, purified AdR, Adx, and CYP11A1 or CYP11B1 were incubated together with NADPH and cholesterol or 11-deoxycorticosterone, respectively, and product formation was monitored by HPLC (Table 1). These experiments showed that mutant Adx-T71E exhibited a 1.4-fold increased product formation compared to Adx-WT, whereas mutant Adx-T71V displayed no significant changes when assayed with CYP11A1; the determined K_M values were all in the same range. Experiments performed with CYP11B1, on the other hand, revealed no significant changes between the wild type and the Adx-T71 mutants, indicating a differential effect of this residue on the interaction between Adx and these cytochrome P450s. Additional in vitro reconstitution assays using CK2 phosphorylated Adx (see Figure 5) validated these findings. For the formation of pregnenolone from cholesterol by CYP11A1, we determined V_{max} to be 6.03 ± 0.63 [nmol pregnenolone/min * nmol CYP11A1] ($P = 0.0051$ versus Adx-WT). These measurements displayed the same activating tendency as the assays conducted with the mutant Adx-T71E and thus support the concept that CK2 phosphorylation of Adx results in increased pregnenolone formation by CYP11A1. These experiments also show that in this case, it is justified to use the substitution of a Thr residue by a Glu for mimicking a phospho-Thr.

Influence of Adx-T71 Mutants on the Steroid Hydroxylase Activity of Bovine CYP11A1 and CYP11B1 after Transient Expression in COS-1 Cells. To determine whether CK2 phosphorylation of Adx has a potential influence on P450-dependent activity in a cellular system, we assayed steroid hydroxylation activity in COS-1 cells transiently cotransfected with expression plasmids for Adx-WT or Adx-T71E and either bovine CYP11A1 or bovine CYP11B1, respectively. These assays were also performed to verify the differential effect of CK2 phosphorylated Adx and the mutant T71E observed in in vitro reconstitution experiments. As mentioned above, CYP11A1 accomplishes the side-chain cleavage reaction that leads from cholesterol to pregnenolone, while CYP11B1 has two natural substrates: It catalyzes the 11β-hydroxylation of 11-deoxycortisol to cortisol, and it also catalyzes a series of three reactions (11β-hydroxylation, 18-hydroxylation, and 18-oxidation) that leads from 11-deoxycorticosterone via corticosterone and 18-hydroxycorticosterone to aldosterone. Since the uptake of cholesterol by mammalian cells is limited because of the lack of the steroidogenic acute regulatory protein (StAR), we used 22R-hydroxycholesterol as a substrate, which bypasses the action of StAR (*40*−*42*).

When CYP11A1 catalyzed formation of pregnenolone from 22R-hydroxycholesterol was monitored, a 1.5-fold increase in substrate conversion was observed with Adx-T71E ($P < 0.001$; Figure 6). This increased product formation can be explained by assuming that mutant Adx-T71E behaves like a 100% phosphorylated Adx, whereas phosphorylation of Adx-WT by CK2 within the cells assumably is not total. By contrast, CYP11B1 activity toward both of its substrates, 11-deoxycortisol and 11-deoxycorticosterone, was the same with both Adx species. The outcome of these assays underlines the results obtained from in vitro reconstitution assays with Adx-T71E suggesting that phosphorylation of Adx at Thr-71 affects its interaction with CYP11A1 but not with CYP11B1.

DISCUSSION

Adrenodoxin (Adx) is an essential component of mammalian mitochondrial cytochrome P450 systems where it plays a crucial role in the biosynthesis of all steroid hormones. Previous studies have shown that Adx is a phosphoprotein (*3*−*5*), but the few published investigations on phosphorylated Adx were at least in part contradictory or unable to be reproduced. Thus, the relevant kinases, the

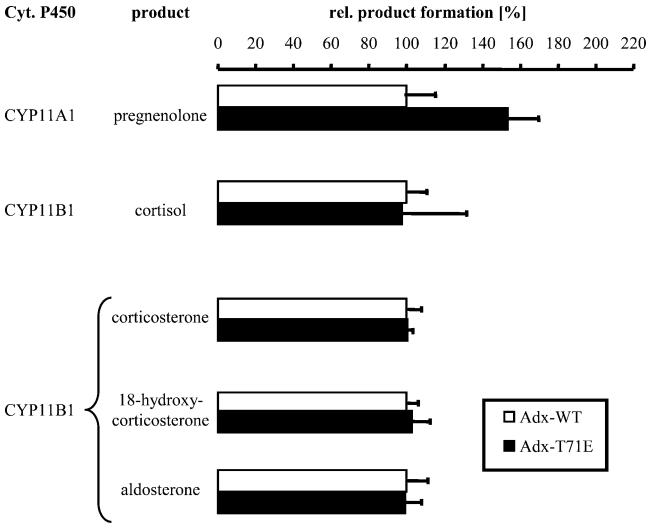

FIGURE 6: Steroid hydroxylation assays in transiently transfected COS-1 cells. COS-1 cells were cotransfected with expression plasmids for a bovine cytochrome P450 steroid hydroxylase (pSVL-bCYP11A1 and bCYP11B1, respectively) and expression plasmids for Adx-WT (pbAdx4; open bars) or Adx-T71E (pbAdx4^{T71E}; black bars) as indicated. Activity of CYP11A1 was determined by measuring pregnenolone production from 22R-hydroxycholesterol; CYP11B1 activity was monitored by measuring 11β-hydroxylation of 11-deoxycortisol to cortisol as well as formation of corticosterone, 18-hydroxycorticosterone, and aldosterone, respectively, from 11-deoxycorticosterone as indicated. Steroids were extracted from cell culture supernatants and analyzed by HPTLC and autoradiography as described in Experimental Procedures. Mock-transfected cells did not display product formation under these conditions (data not shown). Data presented were calculated from three independent experiments.

phosphorylation sites, and the functional consequences of Adx phosphorylation still await a detailed analysis. Here, we demonstrate that bovine Adx is efficiently in vitro phosphorylated by protein kinase CK2 (Figure 1A), while CYP11A1, CYP11B1, and AdR (all bovine) were not phosphorylated (Figure 1B). As an attempt to map the CK2 phosphorylation site on Adx, we tested a set of five 11-amino acid long peptides that contain the putative CK2 phosphorylation motifs of human and bovine Adx, respectively, by incubating each with the CK2 holoenzyme and ^{32}PγATP. However, none of these peptides were efficiently phosphorylated, in contrast to a positive control peptide (DDDDDS-DDDDD) (data not shown). These results suggest that Adx, like most CK2 substrates (*9*), is only phosphorylated by the holoenzyme, but not by the catalytic CKα subunit alone. This notion was confirmed by other experiments as shown in Figure 1C. We identified Thr-71 as the single phosphorylation site in Adx by doing experiments on a series of relevant mutants (Figure 1D). This residue is located on the surface of the protein and part of the AdR−CYP11A1 interaction domain of Adx. Interestingly, the T^{71}-D-E-E motif of bovine Adx is completely conserved in human Adx but not in the fission yeast homologue etp1, which contains the sequence E-E-D-E at this position. Both human Adx and etp1 are also phosphorylated by CK2 (data not shown), but the identification of the CK2 phosphorylation sites in these proteins was out of the scope of this study.

Previous studies suggested that Adx is not a CK2 substrate (*4*). However, kinase purification procedures (including recombinant expression) have dramatically improved since those studies were made permitting more accurate and reproducible results nowadays. To investigate the consequences of Adx phosphorylation by CK2 in detail, we created two Adx mutants that mimic either a completely dephosphorylated state (Adx-T71V) or a completely phosphorylated condition (Adx-T71E) and then studied their functional properties. These data were complemented with additional experiments using the phosphorylated protein. Spectroscopic analysis using CD-, EPR-, and UV−vis measurements as well as the redox potentials of both mutants and the phosphorylated protein are not significantly altered compared to Adx-WT (Figures 3 and 4). These data suggest that the Thr-71 mutations introduce no significant conformational changes in the vicinity of the [2Fe-2S] cluster. Additionally, the symmetry of the reduced cluster remains unaltered, as does the ability of Adx to accept and donate electrons.

The effect of phosphorylation of Thr-71 on the function of Adx is connected to the relative position of this phosphorylated residue with respect to the binding domains of Adx with its redox partners. Although recently new interaction regions comprising an acidic patch around residue Asp-39 as well as the iron−sulfur cluster surrounding loop (residues 47−51) were identified (*12*, *20*), the primary region for redox partner recognition of Adx is the so-called interaction domain, consisting of residues 67−87 (*34*, *43*). Using site-directed mutagenesis and chemical modification studies, the nature of the redox partner recognition mechanism has been unequivocally identified as depending mainly on electrostatic interactions involving the dipole character of Adx (*6*). The charged residues of Adx are clustered and yield a strikingly asymmetric electric potential of the protein molecule, which renders one face of Adx almost completely acidic. This asymmetric charge distribution results in a large dipole moment that may play a biologically relevant role during the docking process of Adx to its electron donor and acceptors (*24*). The negative end of the dipole vector of Adx crosses the molecular surface near the acidic residues Asp-72, Glu-73, Asp-76, and Asp-79 (*44*) that interact with positively charged amino acids of AdR and CYP11A1, respectively. In contrast to the highly conserved core region in all ferredoxins, this interaction domain is a very variable region that determines or modulates the interaction toward the corresponding redox partners (*44*). The comparison of the atomic structures of a complex of Adx with AdR (*45*) to biochemical and molecular modeling studies on the interaction of Adx with CYP11A1 (*46*) has revealed that the binding sites for AdR and CYP11A1 on the Adx surface are overlapping but not identical. For instance, the region between Asp-76 and Asp-79 of Adx represents the primary interaction site with AdR (residues Arg-240 and Arg-244) (*43*) being essential for the formation of a functional complex. In addition to this region, residues Asp-72 and Glu-73, which face away from the AdR/Adx complex interaction site (*45*), are necessary for the proper interaction between Adx and CYP11A1 (*46*−*48*). Since Thr-71 is adjacent to these amino acids, a modification of this residue affecting its charge (see Figure 7) could lead to an enhanced binding to CYP11A1, whereas its interaction with AdR is not expected to change significantly. The optical difference spectroscopy experiments presented here point to an enhanced binding of CK2 phosphorylated Adx. This phosphorylation at residue Thr-71 conceivably increases the acidity of this important interaction region and, thus, facilitates the

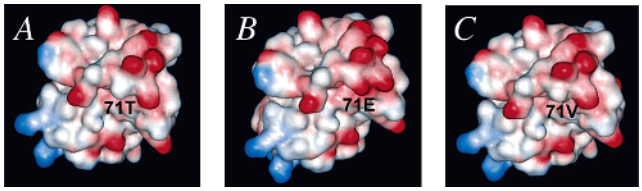

FIGURE 7: Comparison of electrostatic potential surfaces of Adx species. Electrostatic surfaces of Adx-WT (A), Adx-T71E (B), and Adx-T71V (C) were generated using the program WEB-LAB-Viewer (http://www.accelrys.com). Positive charges are displayed in blue color, and red-colored regions indicate negative charges. The substitution T71E, like a phosphorylation at this residue, adds to the acidic character of the part of Adx that interacts with basic regions of CYP11A1.

binding to basic residues K405, K403, and R426 of CYP11A1 (*46*) or even causes residue Thr-71 to participate more directly in the interaction. An enhanced binding of Adx T71E or CK2-phosphorylated Adx to CYP11A1 is very likely to be the cause for the enhanced substrate conversion activity that was observed in in vitro reconstitution assays (Figure 5) and in steroid hydroxylation assays performed in transiently transfected COS-1 cells (Figure 6), whereas in vitro reconstituted steroid hydroxylation experiments using the mutant Adx-T71V did not lead to increased CYP11A1 activity as compared to Adx-WT (Table 1). In contrast to the interaction between Adx and AdR or CYP11A1, little is known about the binding mechanism between the ferredoxin and CYP11B1. Site-directed mutagenesis studies have implied that the CYP11B1/Adx complex formation is more dependent on hydrophobic interactions (*49*, *50*) involving the hydrophobic region of Adx between the iron−sulfur cluster and the acid patch located in the interaction domain. Therefore, this region seems to play an essential role in modulating the formation of a properly orientated and functional complex so it is not surprising that additional charge insertions at position Thr-71 did not severely affect the mode of interaction between these two proteins. The CYP11B1 in vitro and COS-1 cell steroid hydroxylation assays using the Adx mutant T71E as well as in vitro reconstitution assays with the phosphorylated species yielded no changes compared to WT-Adx. Surprisingly, the mutant T71V displayed a slightly altered behavior toward CYP11B1 (Table 1). Nevertheless, these changes are not very pronounced and lie within the range of the standard deviation so they can therefore be neglected. Naturally, the results presented here raise the question whether CK2 phosphorylation of Adx is physiologically relevant or not. Protein kinase CK2 is a ubiquitous kinase that is present in the nucleus, cytosol, microsomal, and mitochondrial fractions (*51*, *52*) as well as in the extracellular matrix (*53*). Taking the present data into account which point toward a differentially altered interaction of phosphorylated Adx as well as Adx-T71E to CYP11A1 and CYP11B1, respectively, it is tempting to postulate a regulatory function of CK2 in steroidogenesis. Nevertheless, the examination of this question was not the aim of this study.

Summarizing, this study identified that only Adx is phosphorylated by CK2 and that Thr-71 is the phosphorylated residue. All experiments presented here indicate that a charge insertion at position T71 leads to a better binding of this protein to CYP11A1 but not to CYP11B1. In addition, even if the V_{max} value for the mutant T71E is only moderately increased (~1.4-fold) as compared to other, for example, C-terminally truncated Adx species, such as the mutant S112W (6.7-fold increased) (*39*), Adx-T71E represents the first full-length mature Adx species that leads to an increase in product formation with CYP11A1 (*6*). Since it has not been shown that Adx species that are degraded C-terminally are physiologically relevant, the findings presented here are important for our understanding on the mode of interaction between the different components of the steroid hydroxylating system as well as on the functional consequences of phosphorylation of iron−sulfur proteins.

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