 Chloro(2,2′:6′,2″-terpyridine) platinum inhibition of the renal Na\textsuperscript{+},K\textsuperscript{+}-ATPase

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The sodium pump is responsible for maintaining the Na\textsuperscript{+} and K\textsuperscript{+} gradients in most animal cells with the uphill transport of Na\textsuperscript{+} and K\textsuperscript{+} fueled by ATP hydrolysis. In the normal cycle, extracellular K\textsuperscript{+}, intracellular Na\textsuperscript{+}, Mg\textsuperscript{2+}, and ATP are the substrates. The transport and ATP hydrolysis cycles involve a series of conformational changes (10, 11).

Chemical modifications have been used previously to study the effect of ligands on conformational changes of the sodium pump (2, 3, 15, 17, 18, 19). In some cases, the chemical modification introduces a fluorescent compound, and the fluorescence is sensitive to changes in its environment. For example, FITC modifies Lys501, and its fluorescence changes upon the addition of Na\textsuperscript{+} and K\textsuperscript{+} (4, 11, 24, 25). Unfortunately, the FITC-modified pump is unable to complete the ATPase cycle. In this regard, iodoacetamidofluorescein (IAF) modification has become more useful, because IAF attachment does not inhibit the pump and its fluorescence does change upon the addition of Na\textsuperscript{+} and K\textsuperscript{+}. Tyson et al. (23) identified the cysteine residue that IAF modifies by isolating and sequencing the labeled peptide. The sequence data suggested that Cys457, the second of two vicinal cysteines, was modified. Consistent with these sequence data, they found that phenylarsine oxide, which specifically modifies vicinal cysteines (8), prevented IAF modification. Interestingly, phenylarsine oxide modification at low concentrations (which prevented most of the IAF incorporation) also did not inhibit the pump.

Often the reactivity of specific residues depends on the conformation, and FITC is an example of this. ATP protects against modification by FITC at Lys501 (11). This finding and other modification data led to the suggestion that Lys501 was part of the ATP binding pocket; this has been confirmed in the sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) crystal structure (22). SERCA is homologous to the sodium pump, and it is likely that many key structural features are conserved in the two pumps (21). Another example of a modification that is conformationally sensitive is that by 2-(4′-maleimidylanilino)naphthalene 6-sulfonic acid (MIANS) (6). MIANS inhibition could be blocked by ATP or by low concentrations of K\textsuperscript{+}. Gatto et al. (6) showed that MIANS modified Cys577 and suggested that ATP protected the residue because it was part of the ATP binding site and that K\textsuperscript{+} protected by allosterically modifying the site. This allosteric effect is consistent with the observation that K\textsuperscript{+}, bound at transport sites, lowers the affinity for ATP by two orders of magnitude.

A high-resolution crystal structure of the sodium pump is not yet available, but Toyoshima et al. (22) have obtained a high-resolution crystal structure of SERCA. SERCA is homologous to the sodium pump, and Sweadner and Donnet (21) have provided a detailed analysis of the similarity of the two structures. Interestingly, the SERCA structure supports the conclusions from the chemical modification studies mentioned above in that the FITC residue, Lys501 in the sodium pump and Lys515 in SERCA, is clearly part of the nucleotide-binding pocket, though it is not yet...
known whether either of these lysines make direct connections to ATP. The MIANS residue, Cys577 (Thr592 in SERCA), is also part of the nucleotide-binding domain but is buried in the structure. The IAF residue, Cys457 (Cys471 in SERCA), is on the exterior of the nucleotide-binding pocket.

Surface-reactive chemical modifiers may be particularly useful conformational probes because the accessibility and reactivity of surface residues might be easily altered by subtle conformational changes. Site-directed mutagenesis allows one to target a change to a specific amino acid residue in the primary sequence; it is difficult a priori to target a chemical modifier to a specific amino acid residue in the primary sequence. However, chemical modifiers work on the properly folded, native structure of their targets, whereas the structure of genetically modified proteins may be profoundly affected by site-directed mutations. It is not feasible to determine structures for all possible mutants. Thus another benefit of chemical modifiers is in studies of site-directed mutants; these modifiers have been and will continue to be necessary as probes of the different sodium pump conformations and to detect subtle changes in conformation.

To broaden the palette of conformational probes used for the sodium pump, we examined the effects of chloro(2,2′:6′,2″-terpyridine) platinum (terpyridine platinum), a novel, water-soluble, positively charged modifier that reacts with histidines and cysteines (12). In a study of photosystem II, it was shown that terpyridine platinum reacted with histidines and that Mn protects photosystem II from this reaction (7). Terpyridine platinum also protected plasmin, a serine protease, from oxidative inactivation (14).

We show here that terpyridine platinum reacts with Cys452, which is on the exterior of the nucleotide-binding pocket. The reaction is accelerated by K+1, whereas vanadate, eosin, Na+, and Mg2+ have no effect on the rate of reaction. ADP was unable to stimulate ouabain binding to terpyridine platinum-modified enzyme. The terpyridine platinum-modified enzyme did not bind [3H]ADP. These results suggest that modification of this residue prevents ADP binding, even though occupancy of the nucleotide-binding pocket (by eosin) does not alter the reactivity of the residue.

**MATERIALS AND METHODS**

Purified canine kidney ATPase was supplied by Gatto as described (6). The method of Jørgensen (9) was followed with the addition of further enzyme purification through a continuous sucrose gradient (15–45% sucrose).

**Time course of terpyridine platinum inhibition of ATPase activity.** The purified canine renal sodium pump enzyme was diluted into media containing 200 mM N-methyl-D-glucamine (NMDG)-HEPES, pH 7.4, and the appropriate sodium pump ligand (e.g., K+, Na+, Mg2+, eosin, or vanadate) in the presence or absence of 4.5 μM terpyridine platinum. After incubation at 37°C for 2, 6, 9, 13, or 25 min, the samples were diluted −10-fold in ATPase buffer containing 1 mM EGTA, 20 mM KCl, 130 mM NaCl, 50 mM sodium-HEPES, 3 mM Tris-ATP, and 3.3 mM MgCl2 and then incubated an additional 10 min at 37°C. The ATPase reactions were then stopped with a twofold dilution into an ice-cold stop solution, and the P1 production was determined colorimetrically as described (1, 6). The experiment included a background ATPase control containing 1 mM ouabain (in quadruplicate) for each condition (presence or absence) of terpyridine platinum treatment.

**V8 and trypsin digestion.** Approximately 175 μg of purified canine kidney sodium pump protein was diluted into 24 ml of 200 mM HEPES buffer, pH 7.4, with NMDG and incubated for 10 min at 37°C with 25 μM terpyridine platinum. After completion of terpyridine platinum labeling, the suspension was spun in a Beckman Optima TL ultracentrifuge for 40 min at 500,000 g at 4°C. Supernatant containing unbound terpyridine platinum was then decanted, and the enzyme pellet was washed once with 50 mM Tris buffer, pH 8.0. For trypsin digestion, the enzyme was then resuspended in a total volume of 0.2 ml of Tris buffer containing 1 mg/ml of TPCK-trypsin ( Worthington Biochemicals), 10 mM CaCl2, and 50 μM phenylmethylsulfonyl fluoride (ICN Biomedicals). Digestion then proceeded at room temperature for 30 min. For V8 digestion, the 175 μg of enzyme were then resuspended in a total volume of 2 ml of V8 buffer (containing 10 μg of V8 protease and 0.175 μg of V8 protease endoproteinase Glu-C obtained from Roche Diagnostics). Digestion proceeded for 18 h at room temperature. After completion of either trypsin or V8 digestion, 6 volumes of methanol were then added, and the suspension was incubated for at least 20 h at −20°C. The prepared sample was sent to Washington University (St. Louis, MO) for mass spectroscopic analysis.

**Phenylarsine oxide treatment.** The following methods were employed to determine how the binding of phenylarsine oxide affected terpyridine platinum inhibition. Approximately 35 μg of purified canine renal sodium pump were diluted into 200 mM NMDG-HEPES (prepared as previously described), pH 7.4, and aliquoted into sample tubes at a concentration of ~1 μg of pump per sample. Phenylarsine oxide (0 or 300 μM or 1 mM in DMSO) was then incubated with the pump for 10 min at 37°C. Because phenylarsine oxide is slowly reversible (23), the 30 μM terpyridine platinum was added to the samples in the presence of phenylarsine oxide and the ATPase activity was determined as previously described. The ATPase samples without phenylarsine oxide contained an equivalent amount of DMSO as a control.

**[3H]Ouabain binding.** The canine renal sodium pump enzyme was treated with control buffer or 40 μM terpyridine platinum as described in **V8 and trypsin digestion** for 5 min. (This concentration of terpyridine platinum was required in paired control experiments at this time to achieve significant ATPase inhibition.) Approximately 12 μg of enzyme were included in each sample for the [3H]ouabain binding and assayed in either quadruplicates (maximal activity controls) or singletons. The samples were assayed in a reaction mixture containing 4 mM MgCl2, with ADP concentrations varying from 0 to 100 μM, in the presence or absence of 1 mM unlabeled ouabain, 3.5 mM Tris·HCl, pH 7.5, and 5 μCi (150 nM) of [3H]ouabain (per experiment). Samples were incubated in the reaction mixture for 60 min at 37°C and then diluted approximately 10-fold into ice-cold 50 mM Tris·HCl, pH 7.5. The diluted samples were placed on ice until the entire volume of each sample was placed over a pre-wetted 0.45-μm cellulose filter (Osmonics). After filtering, the filters were washed three times with 2 ml of ice-cold Tris·HCl. The filters were then counted.

**[3H]ADP binding.** The sodium pump was diluted 38-fold into 25 mM Tris·HCl, pH 7.4, and divided into two aliquots. The aliquots were incubated in the presence or absence of 35 μM terpyridine platinum for 15 min at 37°C. In paired
experiments we found that this higher concentration of terpyridine platinum was required to achieve ~80% inhibition of the ATPase activity. (These experiments were done over 1 yr later than the ATPase experiments.) The samples were then centrifuged at 500,000 g for 5 min. The pellet was resuspended in imidazole buffer and homogenized. (In separate experiments, the trapped space in the pellet was <1%, so the terpyridine platinum concentration would be much less than 0.1 μM.) [3H]ADP was added, and specific binding was determined as the difference between the binding at 2 and 500 μM ADP in the pellet obtained after centrifugation at 500,000 g for 5 min with triplicate samples. A tissue was used to remove all the fluid before the pellet was placed in scintillation fluid.

RESULTS

Terpyridine platinum is a bulky, water-soluble reagent that reacts with cysteines and histidines (15). Figure 1 shows the time course of inactivation of Na⁺,K⁺-ATPase by terpyridine platinum. The time course is fit by a single exponential, consistent with one class of sites modified by terpyridine platinum. In other experiments, we observed 100% inhibition with longer incubations or higher concentrations.

We next set out to determine whether the rate of reaction of terpyridine platinum with the pump would be altered by the addition of different pump ligands.

Terpyridine platinum is positively charged and thus may be directed to a cation-binding site. We determined whether K⁺ would protect the pump from reaction with terpyridine platinum. In contrast to our expectation, we found that K⁺ accelerates the reaction of terpyridine platinum (Fig. 2A). K⁺ is known to have a biphasic effect on the reaction of MIANS and DIDS with the pump (5, 6); at low concentrations it protects and at high concentration it allows the reaction. We determined the concentration dependence of K⁺ on terpyridine platinum reaction (Fig. 2B). In this concentration range, K⁺ only accelerates the reaction, with an apparent EC₅₀ of 1 mM. This EC₅₀ is consistent with K⁺ acting at the intracellular transport site.

Unlike K⁺, Na⁺ did not have any significant effect on the rate of reaction of the pump with terpyridine platinum, as shown in Fig. 3A. The sodium pump also has another cation site, for Mg²⁺, so we determined whether Mg²⁺ would alter the reaction with terpyridine platinum. We found that Mg²⁺ had no effect on the reaction with terpyridine platinum (Fig. 3B).

The pump can bind ATP, K⁺, or vanadate in such a way that the binding of one of these ligands alters the affinity for the others. Because K⁺ altered the rate of reaction with terpyridine platinum, we wanted to determine the effects of ATP and vanadate on the agent’s inhibition of the pump. The presence of ATP in the incubation solution with terpyridine platinum significantly reduced the extent of inhibition observed (data not shown). We determined that terpyridine platinum interacted with ATP as the absorbance spectra of terpyridine platinum (between 300 and 360 nm) changed upon the addition of ATP. This portion of the absorbance spectra is also known to change upon reac-
tion of terpyridine platinum with histidine or cysteine (12). Thus the reduction in pump reaction probably reflects a decrease in the concentration of reactive species of terpyridine platinum. As an alternative to ATP, we determined the effect of eosin, a competitive inhibitor of ATP binding (20), on the ability of terpyridine platinum to inhibit. As shown in Fig. 4, eosin had no effect on the reaction of terpyridine platinum with the pump. This shows not only that terpyridine platinum is not going to the ATP site but also that occupancy of the nucleotide-binding site does not influence the reaction with terpyridine platinum.

To the best of our knowledge, no one has tested the effects of vanadate on FITC reactivity on the sodium pump. For SERCA, which is also a member of the P-type ATPase family, vanadate has been shown to protect against FITC labeling (16). Vanadate is a Pi analog and acts as a product inhibitor of the sodium pump. As expected, we found that vanadate (32 μM) protects the sodium pump against inhibition by FITC (35 μM) (data not shown). We determined that vanadate (32 μM) had no effect on the inhibition by terpyridine platinum (rate constants were 0.067 ± 0.003 and 0.070 ± 0.005 min⁻¹, n = 3). Thus vanadate does not alter the accessibility or reactivity of the terpyridine platinum residue.

To identify the residue modified by terpyridine platinum, we treated terpyridine platinum-labeled pump with trypsin and then subjected the sample to mass spectrometric analysis. Of the masses obtained, only one was consistent with terpyridine platinum modification: 452CIELCGSVKEMRDR. The experiment was also done with terpyridine platinum-modified pump proteolyzed with V8, which cuts after glutamate and aspartate residues. An example of the mass spectrum obtained is shown in Fig. 5. Of the masses obtained, only one was consistent with terpyridine platinum modification: 446SALLKCIELCGSVKEMRDR. It is important to note that both fragments contain cysteines 452, 456, and 457 but no histidines.

IAF modifies the second of the vicinal cysteine residues in the pump, and this modification does not inhibit the pump (23). To determine which cysteine is modified, we took advantage of the approach used in identifying the IAF residue as a similar peptide was labeled in this study. Even though terpyridine platinum and IAF have a similar size, it would still be possible for terpyridine platinum to modify Cys457 and inhibit the pump because of the charge difference between terpyridine platinum and IAF. Alternatively, terpyridine platinum could modify Cys452 or Cys456. Vicinal cysteines react with phenylarsine oxide (8), and phenylarsine oxide modification of these cysteines in the sodium pump does not inhibit the pump (23). If terpyridine platinum modified one of the vicinal cysteines, then phenylarsine oxide pre-treatment should prevent the modification and thus terpyridine platinum inhibition. As shown in Fig. 6, terpyridine platinum still inhibits phenylarsine oxide-modified enzyme. In these experiments, we used enough phenylarsine oxide to inhibit the enzyme partially; thus we can conclude that phenylarsine oxide has modified the vicinal cysteines and, in some pumps, additional cysteines as well. Nevertheless, terpyridine platinum still fully inhibits the phenylarsine oxide-modified enzyme, implying that the...
inhibition is not via the vicinal cysteines but is by exclusion due to Cys452.

We determined whether terpyridine platinum-modified pump could still react with FITC, which is known to react with Lys501. Terpyridine platinum-modified pump significantly reduced FITC labeling of the pump (data not shown). The lack of reaction of terpyridine platinum-modified pump with FITC could be because FITC could not bind to modified pump or because the modification alters the reaction with FITC, for example, by altering the local pK of the Lys501 or by allosterically altering its position so that, even though FITC reversibly binds, the isothiocyanate group is not close enough to the lysine to label irreversibly.

We next determined whether terpyridine platinum-modified pump was able to increase ouabain binding in response to ADP. ADP has been shown to increase the rate of ouabain binding to the pump (13). As shown in Fig. 7, ADP increased ouabain binding in control enzyme with a $K_{1/2}$ of 14 $\mu$M, similar to previously reported values for nucleotide effects on ouabain binding (13) and in the range expected for ADP binding to the high-affinity nucleotide site. For these experiments, we measured $[^3H]$ouabain binding before equilibrium binding was achieved; on the basis of previous results, we infer that the ADP effect on ouabain binding reflects a change of the on rate (and thus also the $K_d$) for ouabain binding, and not the maximal extent of binding. However, we observed no stimulation of ouabain binding by ADP in terpyridine platinum-modified pumps. Terpyridine platinum-modified pumps did bind ouabain, in the absence of ADP, to the same extent as

Fig. 5. Mass spectral analysis of terpyridine platinum-modified sodium pump after V8 digestion. The ExPasy program predicted a peptide of mass 2525.081 for a terpyridine platinum-modified pump fragment after V8 digestion (see Fig. 9A), which is within 0.281 mass units of the observed peak at 2524.801. In the case of the trypsin-digested enzyme, the predicted mass was 2623.002 and the measured mass was 2623.290. The only fragments from mass spectral analysis of terpyridine platinum-modified pump after either trypsin or V8 digest that were consistent using the ExPasy program with terpyridine platinum modification were peptides that contained no histidines and Cys452, Cys456, and Cys457; see text for more details.
control enzyme, indicating that terpyridine platinum did not directly alter the ouabain binding properties of the unliganded pump. These results suggest that modification of this residue prevents either ADP binding or the conformational change following binding.

To determine whether nucleotides could still bind to the terpyridine platinum-modified pump, we directly measured [3H]ADP binding to the pump. As shown in Fig. 8, terpyridine platinum-modified pumps had significantly less [3H]ADP binding than control pumps. These data suggest that the lack of labeling of terpyridine platinum-modified pump with FITC is probably also due to the inability of FITC to bind. Furthermore, the data indicate that terpyridine platinum inhibits the pump, at least in part, by preventing nucleotide binding, even though occupancy of the site by eosin does not alter the reactivity of the residue to terpyridine platinum (Fig. 4).

**DISCUSSION**

In this study, we have examined the modification of the sodium pump by terpyridine platinum. We have found that terpyridine platinum modifies Cys452 and inhibits the pump. Cys452 is part of the nucleotide-binding domain, though it is not part of the binding pocket. Interestingly, even though Cys452 is on the exterior of the nucleotide-binding pocket, modification of Cys452 by terpyridine platinum prevents nucleotide binding. We found that K+ accelerates the reaction but that Na+, eosin (as ATP surrogate), Mg2+, and vandate have no effect, even though these compounds, like K+, have effects on the apparent affinity for ATP.

Evidence that terpyridine platinum labels Cys452. We have determined that terpyridine platinum reacts with Cys452. First, mass spectral analyses of trypsin and V8 digestion of the labeled sodium pump each were only consistent with modification of peptides containing only Cys452, Cys456, and Cys457. Second, terpyridine platinum still inhibits phenylarsine oxide-modified pump. Because phenylarsine oxide is known to bind to the vicinal cysteines (see e.g., Ref. 23), and the only vicinal cysteines in the sodium pump are Cys456 and Cys457, we concluded that terpyridine platinum reacts with Cys452.

Location of Cys452 in the sodium pump. The sodium pump crystal structures are not yet refined enough to determine the location of Cys452 in the crystal structure precisely. The SERCA structure has been obtained at high resolution (22); the sodium pump and SERCA share a great deal of homology (21). We attempted to use the SERCA structure to understand the role of Cys452 in the sodium pump. Unfortunately, as described below, there are three important sequence differences that do not allow a detailed analysis of the role of Cys452. Although there are subtle differences in the structures of SERCA and the sodium pump in this
region, it appears very likely that Cys452 is exposed to the solvent and on the exterior of, not within, the nucleotide-binding pocket.

The three important sequence differences between SERCA and the sodium pump in the region of Cys452 are as follows: 1) In the alignment of Sweadner and Donnet (21), the SERCA structure has an insert near Cys452 (Leu448 of SERCA) in the primary sequence. The alignment in this region is shown in Fig. 9A. In the SERCA structure, this inserted region is on the surface of the nucleotide-binding domain and is essentially exposed to the bulk solution (see Fig. 9B). One can imagine that it can be easily deleted in the sodium pump, except for the fact that Lys451 and Ala470 are not adjacent in the SERCA crystal structure, but they are close, only about 10 Å apart. When this insert is deleted, Cys452 is still not exposed to the bulk solution.

2) The SERCA structure has an insert between sodium pump residues Trp411 and Phe412 (SERCA residues Ala390 to Leu410), and this insert covers Leu448 of SERCA, the residue homologous to Cys452, the terpyridine platinum-modified residue. Once again, one can imagine that this segment can be easily deleted in the sodium pump, except for the fact that SERCA residues Tyr389 and Val 411 are not adjacent in the SERCA crystal structure; indeed, they are about 20 Å apart. If one removes the two inserts from the SERCA structure, the residue homologous to Cys452 is exposed to the bulk solution, as one would expect for a residue that reacts with the bulky, hydrophilic reagent terpyridine platinum.

3) In the SERCA structure Ile384 is very close to Leu448 (within 6 Å); in the alignment of Sweadner and Donnet (21), the homologous amino acid in the sodium pump is a lysine. This puts a lysine immediately very close to the residue that reacts with the positively charged terpyridine platinum, which seems unlikely.

In contrast to our dilemma with the cysteine modified by terpyridine platinum, the cysteines modified by IAF and MIANS are readily accommodated by the SERCA crystal structure. IAF reacts with Cys457

Table 1. Comparison of different residue labels

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<td>Location</td>
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<td>Outside pocket</td>
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<td>Present work</td>
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SERCA has an insert between sodium pump residues Val457 and Cys456 (452–469 of SERCA), shown in yellow, and an insert between sodium pump residues Trp413 and Phe414 (390–410 of SERCA), shown in cyan. In C, these inserts are shown as wire frames.
(Cys470 in SERCA), which is close in the primary sequence, and spatially, to Cys452. It is shown as green in Fig. 9C and is clearly solvent exposed in the SERCA structure. Reaction with IAF does not alter the pump, in contrast to terpyridine platinum. From the SERCA structure, it is difficult to see why a bulky molecule at Cys452 would inhibit but a bulky molecule at Cys457 would not. It may be that the difference in charge between terpyridine platinum (positive) and IAF (negative) accounts for the different inhibition patterns. It may also be that the presence of positively charged terpyridine platinum at Cys452 causes the lysine at the region homologous to Leu384 to move and that this behavior causes the inhibition.

MIANS reacts with Cys577 (6). In the SERCA structure, the homologous residue (Thr592) is clearly close to the ATP pocket, though it is buried and not exposed, which is not surprising because MIANS is hydrophobic. MIANS modification prevents high-affinity ATP binding.

How does this structural information relate to the functional studies? A model in which Cys452 is more accessible or reactive in E2 states and in which terpyridine platinum modification of Cys452 puts the pump in an E2 state accommodates most of our results. The presence of K⁺ increased the rate of reaction and would put the pump in an E2 state. The E2K formation does not have high-affinity nucleotide-binding, and neither does terpyridine platinum-modified sodium pump. E1 conformations, achieved in the presence of buffer alone, Na⁺, eosiin, and perhaps Mg²⁺, did not modify the rate of reaction. In contrast, vanadate is thought to put the pump in an E2 conformation, but vanadate did not alter the rate of terpyridine platinum modification, suggesting that the vanadate bound conformation does not alter the accessibility or reactivity of Cys452 in the same way that the K⁺-bound conformation does. This is a subtle, yet interesting distinction between these two E2 states.

Cys577, in the ATP binding site, behaves differently than Cys452. Gatto et al. (6) demonstrated that MIANS reacts first with Cys577 and that ATP protects this reaction. Importantly, low concentrations of K⁺ protected the pump from reaction with MIANS. A comparison of the effects of ligands on the reactions of terpyridine platinum and MIANS and the fluorescence of IAF-labeled pump is given in Table 1.

In conclusion, we have shown that terpyridine platinum modifies Cys452, that the modified enzyme is unable to bind nucleotide, and that the rate of this reaction is increased by the presence of K⁺ but is unaffected by the presence of Na⁺, Mg²⁺, eosiin, or vanadate. It will be interesting to determine why the modification of the Cys452 by the positively charged terpyridine platinum affects nucleotide-binding differently from the modification of the nearby residue, Cys457, by the negatively charged IAF.

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