Structural Basis for Autoinhibition and Mutational Activation of Eukaryotic Initiation Factor 2α Protein Kinase GCN2*§

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The GCN2 protein kinase coordinates protein synthesis with levels of amino acid stores by phosphorylating eukaryotic translation initiation factor 2. The autoinhibited form of GCN2 is activated in cells starved of amino acids by binding of uncharged tRNA to a histidyl-tRNA synthetase-like domain. Replacement of Arg-794 with Gly in the PK domain (R794G) activates GCN2 independently of tRNA binding. Crystal structures of the GCN2 protein kinase domain have been determined for wild-type and R794G mutant forms in the apo state and bound to ATP/AMPPNP. These structures reveal that GCN2 autoinhibition results from stabilization of a closed conformation that restricts ATP binding. The R794G mutant shows increased flexibility in the hinge region connecting the N- and C-lobes, resulting from loss of multiple interactions involving Arg794. This conformational change is associated with intradomain movement that enhances ATP binding and hydrolysis. We propose that intramolecular interactions following tRNA binding remodel the hinge region in a manner similar to the mechanism of enzyme activation elicited by the R794G mutation.

Mammalian and yeast cells respond to starvation or stress by down-regulating overall protein synthesis, while increasing translation of specific mRNAs encoding transcription factors by down-regulating overall protein synthesis, while increasing translation of specific mRNAs encoding transcription factors. These responses are induced by protein kinases that phosphorylate serine 51 in the α-subunit of translation initiation factor 2 (eIF2α). The eIF2α (αβγ) heterotrimer forms a ternary complex with GTP and methionyl-tRNA^Met, which delivers Met-tRNA^Met to the small ribosomal subunit (1). Thereafter, this 43 S preinitiation complex binds to mRNA and assembles an 80 S initiation complex at the AUG start codon, with hydrolysis of eIF2-bound GTP and release of eIF2-GDP (2). eIF2-GDP is recycled to eIF2-GTP by the guanine nucleotide exchange factor eIF2B. Phosphorylation of eIF2α converts eIF2-GDP into an inhibitor of eIF2B, thereby decreasing ternary complex formation and protein synthesis (1).

Four mammalian eIF2α kinases regulate translation in response to distinct stimuli: GCN2 by amino acid or serum starvation (3–5) and UV irradiation (6), pancreatic endoplasmic reticulum kinase by unfolded proteins (7), PKR by double-stranded RNA in virus-infected cells (8), and heme-regulated inhibitor by hemin deprivation in erythroid cells (9). All four kinases share a conserved protein kinase (PK) domain (in humans, pairwise amino acid sequence identities 28–33%), each with a unique set of regulatory domains (Fig. 1a) coupling eIF2α phosphorylation to particular stresses.

GCN2 is the most widespread eIF2α kinase superfamily member, first identified as an inducer of GCN4, a transcriptional activator of amino acid biosynthetic genes in budding yeast. Four short open reading frames in the GCN4 mRNA leader support ribosomal reinitiation and increased GCN4 translation in response to moderate reductions in eIF2-GTP. Accordingly, eIF2α phosphorylation in amino acid-starved cells induces GCN4 with attendant increases in amino acid production (10). Similarly, activation of GCN2 in stressed mouse cells, or of pancreatic endoplasmic reticulum kinase in cells experiencing endoplasmic reticulum stress, induces translation of the stress-responsive transcription factor ATF4, while reducing overall rates of translation initiation (5). Activation of GCN2 triggers transcriptional rescue via NF-κB signaling in mammalian cells subjected to UV stress (11).

GCN2 is activated in amino acid-starved yeast cells via binding of uncharged tRNA to its histidyl-tRNA synthetase (HisRS)-like domain (12–14) (Fig. 1a). Starvation for any amino acid activates GCN2 (12, 15). The HisRS domain binds various uncharged tRNAs with similar affinities, discriminating only against their aminooacylated forms (14). A ribosome-binding and dimerization domain (RB/DD) at the extreme C-terminal factor 2; PK, protein kinase; HisRS, histidyl-tRNA synthetase; PDB, Protein data bank; r.m.s.d., root mean square deviation; cAPK, CAM-dependent protein kinase; CDK2, cyclin-dependent kinase 2; c-Src, tyrosine kinase c-Src; CAPS0, N-cyclohexyl-2-hydroxy-3-amino-propanesulfonic acid; CHES, N-cyclohexyl-2-aminoethanesulfonic acid; RB/DD, ribosome-binding and dimerization domain; AMPPNP, 5′-adenylyl-β,γ-imidodiphosphate; WT, wild-type; GCN2, general control non-derepressible-2.
terminus is also required for GCN2 activation (16–19). The RB/DD domain interacts with the HisRS and PK domains in vitro, and there is evidence that the RB/DD-PK interaction impedes GCN2 activation by decreasing the affinity of GCN2 for uncharged tRNAs (14, 18). A degenerate kinase-like domain located immediately N-terminal to the PK domain (ψPK domain, Fig. 1a) and the RWD region at the extreme N terminus of GCN2 serve as additional regulatory domains. RWD supports binding of the GCN1-GCN20 complex (20, 21), which interacts with translating ribosomes and may facilitate transfer of uncharged tRNA from the ribosomal decoding site to the HisRS domain (22, 23).

The isolated PK domain of GCN2 is completely inert in vitro, but, remarkably, activity is rescued by single amino acid substitutions at Arg794 (R794G) or Phe542 (F842L). These constitutively activating (GCN2+) mutations bypass the tRNA binding requirement for kinase activation in vivo, and we propose previously that they alter the PK active site in a way that mimics conformational changes induced by interactions with the HisRS or RB/DD domains on tRNA binding (24). The PK domain interacts with an N-terminal segment of the HisRS domain, and mutations in the latter abolished kinase function, without impairing tRNA binding or dimerization of the HisRS domain. Thus, the HisRS and PK domains probably participate in stable intramolecular interactions, with tRNA binding eliciting kinase activation via conformational changes that propagate from the HisRS domain to the PK domain (18). Other GCN2+ mutations have been described, e.g. E803V (25), whose activated phenotype remains dependent on tRNA binding to the HisRS domain (24). It was proposed that these latter mutations lower the threshold concentration of uncharged tRNA required for kinase activation, allowing high-level GCN2 activity in non-starved cells containing basal levels of uncharged tRNA.

To examine the molecular mechanisms responsible for GCN2 autoinhibition and activation by uncharged tRNAs, we determined a series of x-ray structures of the dimeric catalytic domain of Saccharomyces cerevisiae GCN2 in apo- and ATP-bound forms at 3.0- and 2.75-Å resolution, respectively. These structures reveal partial closure of the active site cleft that propagates from the hinge region between the N- and C-lobes of the PK domain. In addition, we determined x-ray structures of the R794G mutant PK domain in apo- and AMPPNP-bound forms at 1.95- and 2.0-Å resolution, respectively. These two structures demonstrate that this activating mutation increases the flexibility of the hinge segment and opens a "molecular flap" that increases the inter-lobe space and accessibility of the enzyme active site. Hence, our work reveals a novel nucleotide gating mechanism via conformational modulation of the hinge region that controls kinase activity. We propose a two-step activation mechanism in which tRNA binding to the HisRS domain leads to a comparable structural remodeling of the hinge region of wild-type GCN2 that facilitates ATP binding. Subsequent autophosphorylation of the activation loop is predicted to facilitate an additional realignment of active site residues necessary for substrate phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation and Crystallization**—The GCN2 fragment (from *S. cerevisiae*) encompassing residues 594–997 with deleted segment 685–767, and the same fragments bearing the D835N or R794G mutations, were expressed in *Escherichia coli* BL21(DE3) cells as N-terminal His6-Smt3 fusion proteins (26) using a PET28b-derived expression vector (both S- and Se-methionine forms). The methionine prototrophic expression vectors with main chain and side chain stereochemical parameters better than average at this resolution limit (overall G value = 0.2). Subsequent structures of GCN2PKWT, GCN2PKR794G, and GCN2PKD835N were determined via molecular replacement using MOLREP (28) with a GCN2PKD835N monomer search model to re-refined to convergence. The structure of GCN2PKWT, GCN2PKD835N, and GCN2PKR794G was determined by calculating difference Fourier syntheses, using the isomorphous structure of the GCN2PK074Rimer. Residues not included in the final structural models frequently correspond to a few residues (773–779) within the kinase insert, a portion of activation loop (863–879), and the C-terminal α6-helix (968–997). Coordinates of a single pore-forming chain (A) from each structure were used for illustrations.

**Kinase Assay**—The purified proteins (GCN2PKWT and GCN2PKR794G) were analyzed for kinase activity as described previously (24) by incubating with 25 μM [γ-32P]ATP (6000 Ci/mmol, Amersham) and 2.5 μM recombinant eIF2α-ΔC purified from *E. coli* in 20 μl of kinase buffer for 20 min at 30 °C. Reactions were stopped by adding 4× SDS-PAGE sample buffer and boiling, resolved by SDS-PAGE, stained with Coomassie Blue, and autoradiographed. The purified proteins (GCN2PKWT and GCN2PKR794G) were expressed in *S. cerevisiae* and were analyzed by native PAGE and Western blots (26) using antibodies against His6 and GST tags (Amersham).

**Data Collection, Structure Determination, and Refinement**—All diffraction data were collected at SGC-CAT (Advanced Photon Source) under standard cryogenic conditions. A single wavelength anomalous dispersion experiment was conducted at an x-ray wavelength near the selenium absorption peak with a single Se-GCN2PKD835N crystal. 48 of 54 possible selenium sites were located. Experimental phases were estimated at 2.6-Å resolution using MLPHARE (28), and improved by density modification and noncrystallographic symmetry averaging using DM (28). Automated electron density map interpretation with MAID (29) yielded an atomic model encompassing an average of 105 main chain and side chain stereochemical parameters better than average at this resolution limit (overall G value = 0.2). Subsequent structures of GCN2PKWT, GCN2PKR794G, and GCN2PKD835N were determined via molecular replacement using MOLREP (28) with a GCN2PKD835N monomer search model to re-refine to convergence. The structure of GCN2PKWT, GCN2PKD835N, and GCN2PKR794G was determined by calculating difference Fourier syntheses, using the isomorphous structure of the GCN2PK074Rimer. Residues not included in the final structural models frequently correspond to a few residues (773–779) within the kinase insert, a portion of activation loop (863–882), and the C-terminal α6-helix (968–997). Coordinates of a single pore-forming chain (A) from each structure were used for illustrations.
massie Blue, and the dried gel was subjected to quantitative phos-
phorimaging analysis. By varying the concentration of eIF2α-ΔC in the
assays (data not shown), we verified that saturating amounts of sub-
strate yield comparable signals in these assays.

Multiple Sequence Alignments and Homology Modeling—Protein se-
quences similar to GCN2PKWT (E-value < 10−4) were identified using
BLAST (31). Multiple sequence alignments were prepared using
CLUSTALX (32), and amino acid conservation was calculated with
BLOSUM62 (33). The homology model of GCN2 HisRS, residues 1034–
1490, was obtained with MODELLER (34) using the structure of histi-
dyl-β-NGA synthetase from Staphylococcus aureus (Protein Data Bank
code 1QEO) residues 13–420 as a modeling template (E value = 3 × 10−11,
squence identity 19%, model score 1.0). Tests with known
structures have shown that models with scores from 0.7 to 1.0 have the
correct fold-at a 95% confidence level (35).

Coordinate Depositions—Refined atomic co-ordinates and x-ray
structure factors for GCN2PKD835N (PDB code 1ZKE), GCN2PKWT
(PDB code 1ZYC), GCN2PKD835N-MgATP (PDB code 1ZYD),
GCN2PKR794G (PDB code 1ZY4), and GCN2PKR794G-MgAMPNP
(PDB code 1ZY5) are available from the Protein Data Bank (www.rcsb.org/pdb).

RESULTS

Crystallization and Structure Determination—Limited pro-
teolysis/mass spectrometry studies of the GCN2 PK domain
(residues 593–998) confirmed the presence of a large (102 res-
idue) proteolytically sensitive insert that is poorly conserved
among GCN2 homologs (Fig. 1b). An optimal E. coli expression
construct, encoding the active kinase domain (residues 594–
997) lacking the insert loop (residues 665–767), was used to pro-
cude various proteins for crystallographic study. Three dis-
inct proteins, including wild-type enzyme (GCN2PKWT) and the
two mutant forms containing the D835N inactivating muta-
tion (GCN2PKD835N) or the R794G activating mutation (GCN2PKR794G),
were purified to homogeneity and crystallized. GCN2PKWT and GCN2PKR794G
were assayed for auto-
phosphorylation and eIF2α kinase activity (see below). Unmod-
ified native GCN2 PK domains containing the insert region
failed to yield crystals under various crystallization conditions.

The residue-numbering scheme used in this article corre-
ponds to the native GCN2 PK domain (Fig. 1b).

The structure of Se-Met GCN2PKD835N was determined at
2.6 Å resolution via single wavelength anomalous dispersion
and noncrystallographic averaging of three homodimers within
the asymmetric unit (final refinement model gave Rwork =
21.1% and Rfree = 25.9% with excellent stereochemistry).
Thereafter, the refined structure of a GCN2PKD835N monomer
was used as a search model for additional structure determi-
nations, including autoinhibited apoenzyme (GCN2PKWT),
a hyperactive mutant form of the apoenzyme GCN2PKR794G,
and a GCN2PKWT-Mg2+ATP complex. Finally, the structure of the
GCN2PKR794G-Mg2+AMPNP complex was obtained via differ-
ence Fourier syntheses using the isomorphous structure of
GCN2PKR794G (Table I and “Experimental Procedures”).

Structural Overview of the GCN2 PK Domain—The GCN2
PK is a typical, bi-lobate kinase fold with the ATP binding
lobe positioned in the hinge region between the two lobes (Fig.
2a). Alignment of our structure of GCN2PKWT with those of
other protein kinase structures in the PDB, performed using
CE (36), shows conformational similarity to human cyclin-de-
pendent kinase 2 (CDK2, PDB code 1P2A, root mean square
deviation (r.m.s.d.) for equivalent Ca atoms = 2.4 Å), human
tyrosine kinase c-Src (c-Src, PDB code 1F1M, r.m.s.d. = 2.4 Å),
human protein kinase B (PKB/Akt2, PDB code 1MRV, r.m.s.d.
= 2.5 Å), murine cAMP-dependent protein kinase (cAPK, PDB
code 1RES, r.m.s.d. = 2.8 Å), and human hematopoietic cell
kinase (hematopoietic cell kinase, PDB code 2HCK, r.m.s.d. =
3.2 Å). The nomenclature used to describe secondary structural
elements of GCN2 PK follows that introduced for cAPK by
Knighton et al. (37).

The smaller N-terminal lobe (N-lobe, residues 599–788) is
preceded by a short α-helix (αA, residues 594–598) and con-
tains a five-stranded (β1–β5), twisted β-sheet with two α-helices
(αB and αC) linking β3–β4 (Fig. 2a). The position of αC is
displaced as seen in the inactive conformation of CDK2 (38),
c-Src, and hematopoietic cell kinases (39–41). The large insert
characteristic of the eIF2α kinase family members occurs be-
 tween β4 and β5. The nucleotide binding “P-loop” (606–611)
linking β1 to β2 is poorly ordered in some copies of the enzyme
within the asymmetric unit.

The larger C-terminal lobe (C-lobe, residues 795–982) con-
taining amino acids implicated in catalysis, activation, and
substrate recognition is predominantly α-helical (αD–αI), and
is connected to the N-lobe by the hinge region (residues 790–
794, Figs. 1b and 2a). The conformation of the Asp-Phe-Gly
(DFG) motif of apo-GCN2PKWT closely resembles that of the
cAPK in its active conformation (PDB code 1ATP). In both
structures, the DFG motif projects into the active site, whereas
the activation loop extends out into solvent (Fig. 2a, magenta
and orange segments, respectively). The 42-residue activation
loop in GCN2 (residues 853–894, Fig. 1b) is longer than is
typically seen in protein kinases.

Approximately 22 residues at the center of the activation
loop (residues 861–882) appear disordered and are invisible
in experimental electron density maps (Figs. 1b and 2a).

Auto phosphorylation sites within the activation segment,
Thr887 and Thr882 (42), are not phosphorylated in any of
the purified proteins used for crystallization (“Experimental
Procedures”). A segment between αF and αH (residues 921–951)
exhibits high B-factors, consistent with disorder. The most
terminal α-helix (αJ, residues 985–999), which serves as a
linker to the HisRS domain, exhibits minimal non-covalent
interactions with the PK domain and is disordered in various
crystalline forms of GCN2. The remarkable level of pairwise
sequence identity (>34% among all eukaryotes, Fig. 1b) and
the pattern of amino acid differences among GCN2 PK do-
 mains from different organisms allow us to conclude that all
known GCN2 proteins share essentially the same three-di-
ensional structure.

GCN2 PK Forms a Symmetric Homodimer—Consistent with
previously published studies indicating that GCN2 functions as
a homodimer (17–19), the GCN2 PK domain repeatedly crys-
tallized as a symmetric homodimer (Fig. 2b,c) independent of
lattice packing arrangements (Table I). On dimer formation,
~2,600 Å2 of solvent-accessible surface area is buried, which is
consistent with a stable homodimer (43). Size exclusion chro-
matographic studies demonstrated that GCN2 PK is dimeric in
solution (data not shown). The dimer interface is composed
equally of hydrophobic and polar side chains, and is stabilized
by ~26 amino acids from each monomer (Fig. 1b; within resi-
dues 594–830) that participate in 22 hydrogen bonding inter-
actions. All but four of these residues are located in the N-lobe.

Given this dimerization interface, the mode of dimeriza-
tion observed in our crystals almost certainly represents the PK
domain dimer found within the dimer of full-length GCN2.

Inactive Conformation of GCN2 PK—The GCN2PKWT
apoenzyme structure exhibits several features characteristic
of autoinhibited kinase conformations with both a displaced helix
αC and a closed bi-lobate conformation. Structural superposi-
tion of the C-lobes of the apo- and Mg2+ATP-bound forms of
GCN2PKWT documents that ATP binding requires opening of
the cleft between the N- and C-lobes. Among known kinase
structures, a murine cAPK ternary complex (with bound ATP
and inhibitor peptide, PDB code 1ATP) exhibits one of the most
closed bi-lobate conformations (44). Apo-GCN2PKWT adopts an
even more compact, closed conformation, with the N-lobe ~4°
FIG. 1. Domain organization of the elf2α kinases and sequence alignment of GCN2 PK domains. a, domain organization of elf2α kinases. See text for descriptions of the regulatory domains flanking the PK domain of GCN2. Residue numbering corresponds to *S. cerevisiae* GCN2. b, secondary structure and sequence alignment of GCN2 PK domains. Secondary structural elements obtained from our GCN2PKWT-Mg,ATP structure are shown as cylinders (α-helices) and arrows (β-strands) above the aligned sequences. Black dots denote disordered residues. Sequence conservation among 21 GCN2 PK domains is depicted with a white → yellow → green color ramp (30 → 65 → 100% identity). Six representative GCN2 PK sequences are displayed with the color ramp, and correspond to *S. cerevisiae*, *N. crassa*, *D. melanogaster*, *A. thaliana*, *M. musculus*, and *H. sapiens*. The kinase insert deletion Δ665–767 lies between the down arrows (↓). Invariant protein kinases residues are taken from Ref. 53.
### Summary of phase determination and crystallographic refinement statistics

| Structure                        | GCN2PK<sub>D835N</sub> Se-Met | GCN2PK<sub>WT</sub> apo | GCN2PK<sub>WT</sub> Mg<sub>2</sub>ATP | GCN2PK<sub>R794G</sub> apo | GCN2PK<sub>R794G</sub> Mg<sub>2</sub>AMPPNP |
|----------------------------------|--------------------------------|-------------------------|--------------------------------|-----------------------------|--------------------------------|
| **Wavelength (Å)**               | 0.97935                         | 0.97935                 | 0.97931                         | 0.97931                     | 0.97931                         |
| **Space group**                  | P<sub>2</sub><sub>1</sub>2<sub>1</sub> | P<sub>2</sub><sub>1</sub>2<sub>1</sub> | P<sub>2</sub><sub>1</sub>2<sub>1</sub> | P<sub>2</sub><sub>1</sub>2<sub>1</sub> | P<sub>2</sub><sub>1</sub>2<sub>1</sub> |
| **Molecules/asymmetric unit**    | 6                               | 4                       | 2                               | 2                           | 2                               |
| **Cell constants**               | <br/>a = 79.6 Å, b = 154.1 Å, c = 157.4 Å <br/><br/>a = 79.9 Å, b = 95.7 Å, c = 175.1 Å <br/><br/>a = 82.1 Å, b = 175.2 Å, c = 47.5 Å <br/><br/>a = 52.8 Å, b = 79.3 Å, c = 146.9 Å <br/><br/>a = 52.6 Å, b = 78.9 Å, c = 146.3 Å | <br/>a = 79.9 Å, b = 95.7 Å, c = 175.1 Å <br/><br/>a = 82.1 Å, b = 175.2 Å, c = 47.5 Å <br/><br/>a = 52.8 Å, b = 79.3 Å, c = 146.9 Å <br/><br/>a = 52.6 Å, b = 78.9 Å, c = 146.3 Å | <br/>a = 82.1 Å, b = 175.2 Å, c = 47.5 Å <br/><br/>a = 52.8 Å, b = 79.3 Å, c = 146.9 Å | <br/>a = 52.6 Å, b = 78.9 Å, c = 146.3 Å | <br/>a = 82.1 Å, b = 175.2 Å, c = 47.5 Å <br/><br/>a = 52.8 Å, b = 79.3 Å, c = 146.9 Å | <br/>a = 52.6 Å, b = 78.9 Å, c = 146.3 Å |
| **Resolution range (Å)**         | 40.00–2.60/2.66–2.60            | 50.00–3.00/3.16–3.00    | 50.00–2.75/2.81–2.75            | 50.00–1.95/2.00–1.95        | 50.00–2.00/2.05–2.00            |
| **Reflections**                  | 1,134,151/112,609               | 385,351/26,813          | 228,449/18,487                  | 452,004/45,568              | 442,569/41,845                  |
| **Completeness (%) (overall/outer shell)** | 98.6/96.7                      | 95.0/92.2               | 99.4/97.0                       | 99.1/97.6                   | 94.5/94.3                      |
| **I/\(I\) (overall/outer shell)** | 0.24/0.74                       | 0.24/0.74               | 0.24/0.74                       | 0.24/0.74                   | 0.24/0.74                      |
| **R_{merge} (\(I\))/(overall/outer shell)** | 0.095/0.59                      | 0.077/0.59              | 0.090/0.65                      | 0.086/0.66                  | 0.086/0.64                      |
| **Figure of merit**              | 0.24/0.74                       | 0.24/0.74               | 0.24/0.74                       | 0.24/0.74                   | 0.24/0.74                      |
| **Refinement statistics**        |                                  |                         |                                 |                             |                                 |
| **Number of protein atoms, ligands, and water molecules in the model** | 12,933 protein atoms, 6 glycerols, and 279 water molecules | 8,586 protein atoms and 14 water molecules | 4,314 protein atoms, 4 Mg<sup>2+</sup> ions, 2 ATPs, and 68 water molecules | 4,295 protein atoms, 2 glycerols, and 234 water molecules | 4,295 protein atoms, 2 Mg<sup>2+</sup> ions, 2 AMPPNPs, and 231 water molecules |
| **R_{work} / R_{free}**          | 0.204/0.252                     | 0.234/0.299             | 0.208/0.270                     | 0.224/0.252                 | 0.234/0.279                     |
| **Root mean square deviations**  |                                  |                         |                                 |                             |                                 |
| **Bond lengths (Å)**             | 0.011                           | 0.009                   | 0.006                           | 0.008                       | 0.009                           |
| **Bond angles (°)**              | 1.4                             | 1.5                     | 1.5                             | 1.5                         | 1.5                             |
| **Ramachandran plot statistics** |                                  |                         |                                 |                             |                                 |
| **Residues in most favored region (%)** | 87.5                         | 76.0                    | 87.0                            | 90.1                        | 88.9                           |
| **Residues in additionally allowed region (%)** | 12.3                         | 22.9                    | 13.0                            | 8.8                         | 10.5                           |
| **Residues in generously allowed region (%)** | 0.1                         | 1.1                     | 0.0                             | 1.1                         | 0.6                             |
| **Residues in disallowed region (%)** | 0.0                         | 0.0                     | 0.0                             | 0.0                         | 0.0                             |
| **Overall G-factor**             | 0.2                             | 0.1                     | 0.3                             | 0.3                         | 0.3                             |

\(a R_{merge} = \Sigma_{hkl} \Sigma_{i} (I_{hkl} - \langle I_{hkl} \rangle)/\Sigma_{hkl} \Sigma_{i} \langle I_{hkl} \rangle).\)

\(b\) Computed with MLPHARE/DM.

\(c R_{work} = \Sigma_{hkl} F_{o}(hkl) - F_{c}(hkl)/\Sigma_{hkl} F_{o}(hkl),\) where \(F_{o}\) and \(F_{c}\) are observed and calculated structure factors, respectively.

\(d\) Computed with PROCHECK.
ATP is shown as an atomic stick figure and the Mg$^{2+}$ ions are denoted with brown spheres. The deleted PK insert and the disordered activation loop are shown as broken lines. The phosphorylation site at Thr$^{887}$ is shown as an atomic stick figure. The DFG motif of the kinase domain that occurs next to the β8-strand on the activation loop is obscured by the Mg$^{2+}$ ions. The surface representation of the GCN2PKR794G-Mg$_2$ATP dimer (individual monomers in cyan and green, respectively) with the dimer axis perpendicular to the page. The green monomer surface is rendered semitransparent and shown with an embedded protein ribbon.

closer to the C-lobe than observed in murine cAPK (Fig. 3a). In contrast, the inter-lobe angle of Mg$_2$ATP-bound GCN2PKWT (−91°) closely resembles that of the ATP-bound cAPK (−92°), with the two N-lobes rotated by ~11° with respect to each other. Examination of our structural superposition of the C-lobes of apo- and Mg$_2$ATP-bound GCN2PKWT shows that in the absence of a conformational change, ATP cannot bind to the apo-conformation of GCN2PKWT (Fig. 3b). We believe that GCN2 autoinhibition can be understood as described next.

Inter-lobe mobility in protein kinases reflects mechanical flexibility of the hinge region, which often contains conformationally less restricted glycines (as in the cAPK hinge). There are no glycines in the hinge of GCN2PKWT (Fig. 1b). The residue equivalent to Gly$^{126}$ of cAPK is Arg$^{794}$, which makes a salt bridge with Glu$^{803}$ that binds to Arg$^{847}$ via a second salt bridge (Fig. 3c). Arg$^{794}$ also accepts a backbone hydrogen bond from the amide nitrogen of Ile$^{843}$. Finally, the amide nitrogen of Arg$^{794}$ interacts with the carbonyl oxygen of Glu$^{792}$, forming an unusual, strained $i \rightarrow i+2$ C=O...H-N hydrogen bond that clamps Asn$^{793}$ in a position that partially blocks ATP entry into the bi-lobal cleft. N-terminal to Glu$^{792}$, Cβ of a conserved Cys$^{791}$ participates in a weakly polar C-H...π interaction with the aromatic ring of conserved Phe$^{842}$ (45). Thus, an ensemble of non-covalent interactions rigidifies the hinge region, resulting in strong coupling of the two PK domain lobes. We suggest that these stabilizing interactions preclude ATP entry into the active site, thereby ensuring that the GCN2 PK domain is only active in the context of uncharged tRNA binding. The closed bi-lobate autoinhibited conformation of the GCN2 PK domain differs from those of all other closed, inactive kinase structures (reviewed in Ref. 46).

**Structure of the Hyperactive GCN2 PK R794G Mutant Suggests a Model for Kinase Activation—**In yeast, two mutations (R794G and F842L) bypass the requirement for tRNA binding to the HisRS domain, yielding constitutively active forms of GCN2 in vivo. Whereas the isolated wild-type PK domain is completely inactive, a double mutant form of the enzyme (R794G,F842L) is >500 times more active in phosphorylating eIF2α in vitro versus wild-type (24). Autophosphorylation experiments performed using GCN2PKR794G and GCN2PKWT prepared for crystallization show that the R794G mutation increases autophosphorylation activity ~75-fold (Fig. 4a). GCN2PKR794G is also significantly more active for eIF2α phosphorylation. We could not quantitate the extent of activation because GCN2PKWT is completely inert for substrate phosphorylation (Fig. 4a). Hence, GCN2PKR794G provides a useful model for the activated form of an eIF2α kinase.

Our structure of apo-GCN2PKR794G demonstrates relief of the closed autoinhibitory conformation when compared with that of GCN2PKWT (see supplemental materials Table I for r.m.s.d. values). As shown in Fig. 4b, both GCN2PKR794G structures (apo- and Mg$_2$AMPPNP-bound) closely resemble the relatively open conformation of GCN2PKWT bound to ATP. Mutation of Arg$^{794}$ to glycine, the residue type found at that position in cAPK, disrupts multiple interactions that rigidify the hinge of GCN2 (Fig. 3c), giving rise to both short- and long-range structural perturbations of the PK domain. The conformational freedom of a glycine at position 794 eliminates the strained $i \rightarrow i+2$ hydrogen bond described above, allowing Asn$^{793}$ to extend away from the ATP binding cleft in GCN2PKR794G (Fig. 4, b-d).

Substantial backbone conformational changes occur for hinge residues Glu$^{792}$-Gly$^{794}$ in the GCN2PKR794G structure (Fig. 4, c and d, and supplemental materials Table II). The backbone torsion angles of G794 ($\phi, \psi = 105^\circ, 175^\circ$) adopt a conformation disallowed for non-glycine residues (47). Asn$^{793}$, flanked by Glu$^{792}$ and Gly$^{794}$, extends away from the active site toward the exterior of the PK domain, fully exposing the ATP binding cleft to solvent. A permissive Asn$^{793}$ conformation would provide an energetically favorable state for nucleotide binding, without the need for further opening of the inter-lobate space by domain movement (s). Comparison of the struc-
The activating effects of the F842L (24) and E803V (25) can also be explained by increased hinge flexibility. Replacement of the aromatic side chain of Phe842 with that of leucine would weaken the network of interactions that rigidify the hinge, thereby increasing nucleotide accessibility to the bilobate space. Alternatively, it is possible that hinge loosening in the R794G mutant form of the enzyme enables more dynamic inter-lobe movement, permitting ATP entry to the active site.

Importantly, the activating effects of the F842L (24) and E803V mutations (25) can also be explained by increased hinge flexibility. Replacement of the aromatic side chain of Phe842 with that of leucine would weaken the interaction with Cys791 (45). Combining this change with the R794G mutation would further reduce hinge rigidity, which could explain why a R794G,F842L double mutation produces greater activation of GCN2 than either single mutation (24). The E803V mutation would eliminate the salt bridge between Glu803 and Arg794 that also rigidifies the hinge in wild-type GCN2 (Fig. 3c).

The simplest explanation for the fact that the R794G and F842L mutations bypass the requirement for uncharged tRNA binding to kinase activation is that these mutations mimic structural alterations of the wild-type PK domain resulting from tRNA-binding to the HisRS domain. In contrast, the activated phenotype produced by the E803V mutation depends on tRNA binding to the HisRS domain (24), albeit at basal levels of uncharged tRNAs. Thus, it appears that loss of the Glu803–Arg794 salt bridge does not activate kinase function directly, but does reduce the threshold for tRNA binding to the HisRS domain required to fully relieve the autoinhibitory conformation of the hinge, yielding activation under non-starvation conditions. Although residues Asn793, Arg794, and Glu803 are not highly conserved in organisms other than yeast, we believe that similar but different constellations of polar/charged residues in these positions would secure the hinge via analogous interactions and provide the same overall mechanism of autoinhibition.

**ATP Binding to GCN2 PK—** ATP binding to GCN2 PK exhibits both similarities and differences in protein-ligand interatomic interactions as compared with other protein kinases. ATP binds in a deep pocket, making canonical contacts with conserved residues via the adenine base to main chain atoms of the hinge region between the two lobes (Glu789–Cys791). As in other kinases, the adenine-ribose moiety of ATP resides in the conserved hydrophobic pocket (lined with residues Leu605, Val613, Ala626, and Phe842) and makes water-mediated hydrogen bonds with the “gatekeeper” residue (48 Met788 (Fig. 5a)). Similar adenine-ribose interactions support AMPNP recognition by GCN2PKR794G (Fig. 5b). However, considerable differences are seen in triphosphate recognition by GCN2PK WT and GCN2PK R794G versus other PKs. These differences could be attributed to a paucity of interactions with the P-loop and the displaced location of the α-helix.

Orientation of ATP β- and γ-phosphates in other protein kinases is achieved by repositioning the P-loop within the N-lobe (49). Conversely, binding of ATP to GCN2PK WT and AMPNP to GCN2PK R794G yield similar P-loop conformations that preclude interactions between the P-loop and β- and γ-phosphates (Fig. 5). For GCN2PK WT, the C-lobe alone stabilizes these two phosphate groups, both directly and via co-ordination of two Mg2+ ions (Fig. 5a). In comparison, Mg2+AMPPNP bound to GCN2PK R794G exhibits fewer contacts between the phosphates and the C-lobe (Fig. 5b), possibly because of disorder (static or dynamic) of the bridging metal ion.

**Anchoring of α- and β-phosphate groups is essential for kinase activity.** A conserved lysine residue (from strand β3) typically facilitates their proper orientation (49). In cAPK, conserved Lys628 from strand β3, which is stabilized by Glu91 from helix αC, positions the α- and β-phosphates of ATP in the proper orientation for catalysis (49). In the structures of GCN2PK WT-Mg2+,ATP and GCN2PK R794G-Mg2+AMPPNP, the equivalent basic residue (Lys628) is separated from Glu842 (corresponding to Glu91 in cAPK) by ~11 Å. Instead of the classical ion-pair interaction, Glu842 forms an inter-lobe salt bridge with Arg844 (the Arg of the HRD motif), indirectly linking two invariant residues found in the N- and C-lobes, respectively (Fig. 5). The correct re-orientation of the ATP triphosphates requires significant conformational changes that involve both the P-loop and αC-helix. Next, we propose that these changes are triggered by autophosphorylation of the GCN2 PK activation loop in wild-type GCN2.
DISCUSSION

Implications for the "Second Step" of GCN2 Activation—The activating mutations R794G and F842L lead to increased autophosphorylation of the GCN2 activation loop (Fig. 4a), and there is genetic evidence that phosphorylation of both Thr887 and Thr882 in this loop is required for full activity even in the presence of these GCN2 mutations (24). We propose a two-step activation mechanism for GCN2 in which conformational alteration(s) of the hinge region induced by uncharged tRNA binding to the HisRS domain (step 1) would permit productive binding of ATP to the active site and autophosphorylation of the activation loop (step 2). Our structural analysis suggests that autophosphorylation of Thr887 and Thr882 leads to maximal GCN2 kinase activity by a mechanism observed throughout the "RD" subclass of kinases, wherein electrostatic neutralization of the phosphoresidue(s) drives conformational reorientation of a cluster of basic residues (49). One basic residue that would interact with phosphorylated Thr887 or Thr882 is the invariant RD arginine (Arg834) that precedes the invariant Asp835 (49). In GCN2, Arg834 makes a salt bridge with Glu643, thereby stabilizing the orientation of the displaced α-helix observed in our structures (Fig. 5, a and b).

By analogy with the structures of activated cAPK and CDK2, the conserved Arg834 (Arg165 in cAPK and Arg126 in CDK2) of GCN2 is almost certainly involved in phosphoresidue recognition, which would release Glu643 toward the catalytic site via axial rotation of the α-helix by ~90° with a minimal translation (~3 Å) would position Glu643 near Lys628, creating a salt bridge. An invariant Leu856 residue, near the DFG motif, blocks such a rotation (Fig. 5). Interestingly, an Glu–Arg salt bridge similar to that of the Glu643–Arg834 pair in GCN2 contributes to autoinhibition of c-Src and hematopoietic cell kinase (39–41).

In CDK2, the α-helix is amphipathic in character, whereas it is hydrophobic in the GCN2 PK. The GCN2 α-helix contrib-

FIG. 4. Structure and activity relationships of GCN2PKR794G. a, GCN2PKR794G phosphorylates eIF2α in vitro. The reactions of kinase assay were performed as described under "Experimental Procedures." The protein concentrations are indicated on the top of each panel. Autophosphorylated GCN2PK (P-GCN2PK) was subjected to quantitative phosphoimage analysis (top panel). SDS-PAGE-resolved proteins were stained with Coomassie Blue to show the input GCN2PK and substrate eIF2α-ΔC (bottom panel). Comparing GCN2PK WT at 1000 nM with GCN2PKR794G at 100 nM shows that the autokinase activity of GCN2PKR794G is about 75-fold higher than that of GCN2PK WT. No eIF2α kinase activity was detected for GCN2PK WT. Not surprisingly, given the dimeric nature of the GCN2 protein kinase domain, autophosphorylation is more efficient than eIF2α-ΔC phosphorylation. The use of a truncated recombinant eIF2α subunit (eIF2α-ΔC) instead of full-length multidomain eIF2 as substrate also may have reduced the efficiency of substrate phosphorylation. b, Co backbone views of superimposed wild-type and mutant GCN2 PKs in apo- and ATP/AMPPNP-bound forms. c, conformational changes within the hinge between apo-GCN2PK WT (magenta) and apo-GCN2PKR794G (cyan), with residues 792–794 shown as atomic stick figures. N-lobe and C-lobe surfaces are shown (gray). Asn793, the molecular flap, partially occludes the ATP binding cleft in wild-type GCN2. d, opening of the Asn793 flap exposes the ATP binding cleft in apo-GCN2PKR794G, accommodating the superimposed AMPPNP with no steric clash. AMPPNP is shown as an atomic stick figure within the yellow translucent surface, apo-GCN2PKR794G as a solid gray surface with location of Asn793 indicated in cyan.
stick representation. Met788 (as catalytic Lys628. Hydrophobic interactions stabilizing the inaccessibility by Leu856. In summary, we propose that structural rearrangement induced by autophosphorylation, involving breakage of the individual dimer axes with a common dimer axis and then minimizing the distance between HisRS2 and PK2. HisRS2 was rotated about the common dimer axis so as to optimize shape-complementarity and minimize steric clashes in the putative dimer-dimer interface (Fig. 6). The predicted tRNA binding surface in each GCN2 HisRS monomer, as inferred from the bacterial HisRS-tRNA models (51, 52), is freely accessible in our PK2HisRS2 model. Thus, tRNA binding would not dissociate the PK2 and HisRS2 dimer (Fig. 6c). Importantly, multiple residues in the PK hinge occur in the predicted dimer-dimer interface of the PK2HisRS2 model, suggesting that a conformational change in the HisRS domain elicited by tRNA binding could be transmitted directly to the PK hinge.

Regulation of GCN2 Catalytic Activity—Genetic and biochemical evidence that the R794G mutation bypasses the requirement for tRNA binding for kinase activation, combined with the structural data indicating that it increases hinge flexibility, provides a strong inference that tRNA binding overcomes hinge rigidity as a means of activating GCN2. Residues 750–810, which encompasses the hinge, interact directly with HisRS in our model of the PK2HisRS2 dimer. We suggest that under starvation conditions, binding of uncharged tRNA to the upper surface of PK2HisRS2 (as depicted in Fig. 6c) causes a structural change in HisRS2 that propagates to the hinge regions of one or both PK domains. Remodeling of the hinge would switch the PK domain from the closed, autoinhibited state to the relieved, active conformation, in a manner analogous to the effect of the R794G mutation (Fig. 4c). Although this modeling exercise is not a substitute for an experimental structure of PK2HisRS2, it is notable that residues 1370–1392 of HisRS occur within or near van der Waals contact distance of the PK hinge.

Interestingly, the PK residues altered in the GCN2*-E601K and GCN2*-E821K activated alleles (25) (Fig. 1b) also map to the interface between the PK2 and HisRS2 dimers in our model. These two mutations resemble the E803V mutation, which requires tRNA binding for realization of an activated phenotype (24). Thus, they may directly alter the PK-HisRS interface in a way that lowers the tRNA occupancy of the HisRS domain required to fully rearrange the PK hinge. However, there is evidence that PK-HisRS interactions also impede tRNA binding (18), presumably to prevent kinase activation at low levels of uncharged tRNA. Thus, alterations of the dimer-dimer interface could increase the affinity of the HisRS domain for tRNA as an alternative means of achieving kinase activation in non-starved cells.

The E803V mutation disrupts interaction(s) between RB/DD and PK and increases the affinity of GCN2 for uncharged tRNAs (14), indicating a negative effect of PK-RB/DD interaction on tRNA binding. In our model of PK2HisRS2, the side chain of Glu803 is exposed to solvent where it could interact with the RB/DD domain. As noted above, Glu803 also interacts with a key hinge residue, Arg794, and could mediate an allo-
structural effect of the RB/DD on PK activity. Mutation of the gatekeeper residue, M788V, is unlikely to affect interactions of the PK domain with either of the HisRS or RB/DD domains and probably increases access to the ATP binding cleft instead. In fact, this mutation partially overcomes the requirement for tRNA binding (24), as would be expected if it mimics the effect of the HisRS domain by facilitating ATP binding. Finally, it could be proposed that domain interactions within GCN2 would correct the displaced conformation of α-helix as an alternative mechanism of kinase activation. However, we favor the idea that remodeling the hinge region through tRNA binding would still be required for kinase activation.

Conclusions—The structure of the wild-type GCN2 PK domain presented here provides an explanation for the mechanism of autoinhibition. The closed bi-lobate conformation imposed by a conformationally rigid hinge segment serves as an impediment to ATP binding and catalysis by the wild-type kinase domain. Comparing the structures of the activated R794G mutant and wild-type enzymes provides detailed molecular insights into the mechanism of GCN2 kinase activation via lobe opening and exposure of the ATP binding cleft, resulting from modest perturbations in the hinge. Elucidating the molecular mechanism of kinase activation by determining the structure of a mutant-activated enzyme is a unique aspect of our work. This mechanism also explains the strong activating effects of the F842L mutation, which should increase hinge flexibility, and of the gatekeeper residue mutation, M788V, that should increase ATP access to the active site. Our structure further provides support for a two-step activation process for GCN2 in which remodeling of the hinge, with attendant ATP binding, is followed by autophosphorylation of the activation loop to permit formation of the critical Glu643-Lys528 salt bridge via re-orientation of the α-helix. The PK-HisRS dimer model provides a plausible explanation for tRNA-dependent activation of kinase activity, as the hinge region of the PK domain contributes to the predicted PK-HisRS interface. Hence, a conformational change in the HisRS region upon tRNA binding could be transmitted to the HisRS-PK interface and elicit remodeling of the hinge required to overcome autoinhibition. This process would serve as an efficient signal-transduction mechanism for coupling eIF2α phosphorylation to amino acid starvation in the cell.

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