New Insights into the Autoinhibition Mechanism of Glycogen Synthase Kinase-3β

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Introduction  
Glycogen synthase kinase 3 (GSK-3) is a ubiquitously expressed and evolutionarily conserved serine threonine protein kinase. GSK-3 phosphorylates key signal transduction molecules and is a critical player in metabolism, programmed cell death, and embryonic development.¹⁻³ The enzyme is a promising target for drugs to treat various pathological disorders, including diabetes, neurodegenerative diseases, and psychiatric disorders.³⁻² Unlike many protein kinases, GSK-3 is constitutively active in resting cells and undergoes rapid inhibition by extracellular stimuli.⁸,⁹ The inhibition of GSK-3 is characterized by the phosphorylation of an N-terminal serine residue (Ser9 in GSK-3β and Ser21 in GSK-3α).⁸ An additional unique feature of GSK-3 is that it requires prephosphorylation of its substrates: That is, the catalytic activity is coupled to the binding of phosphorylated substrates that contain the recognition motif for GSK-3, SXXXS(p), where S(p) is a phosphorylated serine.⁴⁻¹⁰ This raised the possibility that the phosphorylated N-terminal tail functions as a pseudosubstrate.  

Protein kinases may be autoinhibited by a mechanism in which part of their polypeptide chain, termed a pseudosubstrate, occupies the active site. Pseudosubstrates are characterized by their resemblance to sequences derived from the substrate target phosphorylation sites and have been identified in cAMP-dependent protein kinase, protein kinase C, and
myosin light-chain kinase. Some experimental findings suggest that GSK-3 is autoregulated by a pseudosubstrate domain. For example, analyses of GSK-3β X-ray structure showed that a phosphate-like moiety interacted with a defined phosphate binding pocket within the catalytic domain, presumably mimicking the interaction with the phosphorylated substrate. Furthermore, it was shown that a synthetic, phosphorylated peptide derived from the potential pseudosubstrate sequence inhibited the kinase. The inhibition was suggested to be specific as it depended on the sequence of the phosphorylated peptide. However, the molecular basis underlying the interaction between the N-terminal pseudosubstrate region and the catalytic core of GSK-3β is not fully defined. It is most likely that sites other than the phosphorylated Ser9 are important for the pseudosubstrate binding and autoregulation. Importantly, the N-terminus of GSK-3β also undergoes autophosphorylation at Ser9. Autophosphorylation and autoinhibition require different binding modes of the N-terminus into the active site of the kinase. In this study, we focused on the binding mode and specificity of the N-terminus as an autoinhibitor, which is likely to resemble the binding specifics of phosphorylated substrates.

We previously showed that Phe67, Gln89, and Asn95 located within GSK-3β catalytic core are important for substrate binding and recognition. We speculated that one or more of these residues may serve as docking sites for the pseudosubstrate. Using molecular, biochemical, and computational analyses, we provide evidence that the pseudosubstrate associates with the active site. We demonstrate that two arginine residues near the N-terminus are critically important for the pseudosubstrate binding and autoinhibition and identify their sites of interaction within the catalytic core.

Results and Discussion

Features of GSK-3 pseudosubstrate region

Analyzing all publicly available sequences of the N-terminal pseudosubstrate region in GSK-3β, GSK-3α, and their orthologs in invertebrates, we found it to be well conserved and present in most GSK-3 family members (Fig. 1a). This included the simplest multicellular animals such as sponges and the unicellular choanoflagellates, the closest known relatives of multicellular animals. Strikingly, Arg4 and Arg6 were absolutely conserved in all GSK-3 pseudosubstrate regions we identified. Ser9 was also highly conserved with only one protein, a GSK-3 from a hydra, where it was replaced by a threonine that can functionally replace serine (as it can be phosphorylated). The analysis further indicated that positions 3–11 of the pseudosubstrate sequence were more conserved relative to the distal positions, suggesting that these nine residues likely comprise the pseudosubstrate core of GSK-3. In addition and independently, we compared the pseudosubstrate domain of GSK-3β with the phosphorylation motif of a known GSK-3 substrate CREB (cAMP response element binding protein). The comparison aligned Ser9 in GSK-3β with the prephosphorylated position in CREB, Ser133 (Fig. 1b). This alignment showed that Arg6 and Phe10 (GSK-3β) were aligned with Arg130 and Tyr134 of CREB, respectively (Fig. 1b). The GSK-3 phosphorylation site, Ser129, in CREB was aligned with Pro5 in GSK-3β. The importance of polar/charged residues in GSK-3’s substrate recognition also suggested that Arg4 and Glu12, both located in proximity to the SXXXS motif (Fig. 1b), may be important sites in this regard. Altogether, our analysis prompted us to investigate the role of Arg4, Pro5, Arg6, Phe10, and Glu12 in the inhibitory capability of the pseudosubstrate.

Arg4 and Arg6 are important sites for pseudosubstrate function

The selected sites Arg4, Pro5, Arg6, Phe10, and Glu12 were mutated to alanine (termed here R4A, P5A, R6A, F10A, and E12A, respectively). The mutant S9A in which Ser9 was mutated to alanine served as a “reference” control. Wild-type (WT) GSK-3β and the various mutants were expressed in a rabbit reticulocyte lysate translation system (TNT) as shown in Fig. 2a. The translated proteins were immunoprecipitated from the reaction mixture with a specific antibody, and GSK-3 kinase assays were performed with p9CREB peptide as substrate. As shown in Fig. 2a, R4A, R4A and R6A exhibited enhanced kinase activity as compared with WT-GSK-3 (increase of 40%). This enhanced activity was comparable to that obtained with S9A. On the other hand, mutations at Pro5, Phe10, and Glu12 did not affect the kinase activity (Fig. 2a). Notably, mutagenesis of Pro5 to Ala mimicked the mammalian GSK-3α sequence; thus, it was expected that this mutation will not affect the catalytic activity. Altogether, Arg4 and Arg6 appear to regulate the catalytic activity, presumably via their ability to interact with residues within the vicinity of the catalytic site.

The N-terminus of GSK-3β can undergo autophosphorylation at Ser9. Since this mechanism requires an interaction between the N-terminus pseudosubstrate and the catalytic core, we examined whether Arg4 and Arg6 are also involved in autophosphorylation. WT-GSK-3 and its respective mutants were subjected to in vitro kinase assays with or without ATP. The reactions were separated by gel electrophoresis and subjected to immunoblot analysis with GSK-3-anti-phospho (Ser9) antibody. Unlike WT-GSK-3, R4A and R6A were unable to autophosphorylate at Ser9 and behaved like the inactive mutant K85MVA (Fig. 2b). This further indicated that Arg4 and Arg6 facilitate the pseudosubstrate “folding” into the catalytic core.
Arg4 and Arg6 facilitate autoinhibition

Next, we investigated the properties of GSK-3 mutants R4A and R6A after expression in cells. HEK-293 cells were transiently transfected with WT-GSK-3 and mutated constructs. The levels of expression are shown in Fig. 3a. As Tyr216 phosphorylation by GSK-3 is mainly an autophosphorylation event and
an indicator for kinase activity.23,31,32 the phosphorylation of Tyr216 was also determined (Fig. 3a). Results indicated that levels of Tyr216 phosphorylation by R4A and R6A were comparable with that of WT-GSK-3, indicating that mutations at Arg4 and Arg6 did not impair the catalytic activity of the kinase. Next, kinase activities toward p9CREB of WT and mutants were tested. The GSK-3 proteins were immunoprecipitated from the cell extracts and were subjected to in vitro kinase assays with p9CREB peptide substrate in the presence of [γ-32P]ATP as described in Experimental Procedures. Reactions were spotted on p81 paper and counted for radioactivity. Results show phosphorylation of p9CREB and are presented as a percentage of WT-GSK-3 activity. Results are the mean of six independent experiments ±SEM. P < 0.01 for mutants S9A, R4A, and R6A versus WT. (b) Experiments were performed as described in (a) except that reactions were performed in the absence of substrate and with nonradioactive ATP. Reactions were stopped with SDS sample buffer and subjected to gel electrophoresis, followed by immunoblot analysis with αpGSK-3 (ser9) antibody. K85,86MA represents an inactive GSK-3 mutant. The gel shown is representative of three independent experiments.

Fig. 2. Protein kinase activity and autophosphorylation of GSK-3β N-terminal mutants. (a) WT-GSK-3 and the various mutants were produced by a coupled transcription/translation system as described in Experimental Procedures. Expression levels of the proteins as determined by Western blot analysis are shown in the lower panel. The various GSK-3 proteins were immunoprecipitated from the reaction mixtures and were subjected to in vitro kinase assays with p9CREB peptide substrate in the presence of [γ-32P]ATP as described in Experimental Procedures. Reactions were spotted on p81 paper and counted for radioactivity. Results show phosphorylation of p9CREB and are presented as a percentage of WT-GSK-3 activity. Results are the mean of six independent experiments ±SEM. P < 0.01 for mutants S9A, R4A, and R6A versus WT. (b) Experiments were performed as described in (a) except that reactions were performed in the absence of substrate and with nonradioactive ATP. Reactions were stopped with SDS sample buffer and subjected to gel electrophoresis, followed by immunoblot analysis with αpGSK-3 (ser9) antibody. K85,86MA represents an inactive GSK-3 mutant. The gel shown is representative of three independent experiments.

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Autoinhibition of GSK-3β is facilitated by the phosphorylation at Ser9, which presumably interacts with the phosphate binding pocket in the catalytic core.16,17 We examined whether Arg4 and Arg6 are important for pseudosubstrate autoinhibition mechanism. To address this question, we treated cells with the phosphatase inhibitor, okadaic acid, to enhance Ser9 phosphorylation and then determined its impact on GSK-3 kinase activity. We first verified that okadaic acid enhanced Ser9 phosphorylation of WT-GSK-3, R4A, and R6A mutants (Fig. 3c). The GSK-3 proteins were then immunoprecipitated from cell extracts and were subjected to in vitro kinase assays. Treatment with okadaic acid reduced WT-GSK-3 activity by 38% (Fig. 3c). However, R4A and R6A mutants were only slightly inhibited by okadaic acid (12.5% and 9.5%, respectively). Thus, although Ser9 was phosphorylated in R4A and R6A mutants, the autoinhibition ability of these mutants was impaired. This indicated that Arg4 and Arg6 enhance the binding affinity of the pseudosubstrate to the catalytic core: Their absence weakened this interaction, resulting in impaired autoinhibition. These results suggest a rational explanation of why R4A and R6A had higher kinase activity than S9A (Fig. 3a). Namely, Arg4 and Arg6 enable a certain degree of binding even in the absence of phosphorylated Ser9.

In summary, Arg4 and Arg6 are involved in the mechanisms of autophosphorylation and autoinhibition. However, it should be emphasized that these two processes require different binding modes to the kinase. Thus, in autophosphorylation catalysis, the nonphosphorylated Ser9 must reside next to the kinase P-loop and the γ-ATP phosphate in order to be phosphorylated, whereas, in autoinhibition, the prephosphorylated Ser9 is positioned in the primed phosphate binding site of GSK-3β. Apparently, Arg4 and Arg6 play a role in both mechanisms and presumably perform different modes of interactions. The following studies focused on the binding mode and specificity of the N-terminus as an autoinhibitor, which is likely to resemble the binding of prephosphorylated substrates.

Gln89 and Asn95 facilitate pseudosubstrate binding and autoinhibition

In a previous study, we showed that Gln89 and Asn95, conserved residues within GSK-3β catalytic core, are important for docking of GSK-3substrates.22 The polar nature of Gln89 and Asn95 enables them to form hydrogen bonds with polar/charged residues in the various substrates.22 We thus speculated that Gln89 and Asn95 should also play a role in pseudosubstrate binding with the catalytic core. As shown in Fig. 4a, mutation at either residue or both impaired the ability to phosphorylate GSK-3 substrate p9CREB. Yet, all mutants were phosphorylated at Tyr216, indicating that their catalytic activity was not impaired (Fig. 4a). We next examined how phosphorylation at Ser9 affected the protein kinase
activity of the mutants Q89A and N95A. HEK-293 cells were transiently transfected with WT-GSK-3, Q89A, and N95A constructs. The cells were treated with okadaic acid to enhance Ser9 phosphorylation. Figure 4b shows that all proteins were phosphorylated at Ser9 in the presence of okadaic acid. The WT-GSK-3 and mutants were then immunoprecipitated from cell extracts and were subjected to in vitro protein kinase assays. Treatment with okadaic acid inhibited WT-GSK-3 kinase activity, but it did not affect that of Q89A or N95A (Fig. 4b). This indicated that both Gln89 and Asn95 are required for the autoinhibition produced by the pseudosubstrate. Phosphorylation at Ser9 and the interaction of this residue with the phosphate binding pocket is not sufficient to confer inhibition; additional interactions supported by Gln89 and Asn95 are required for efficient autoinhibition. To further verify that Gln89 and Asn95 interact with the pseudosubstrate, we subjected Q89A and N95A to in vitro kinase assays in the presence of a short phosphorylated peptide derived from the pseudosubstrate sequence (RPRTTSpFAES). This peptide was previously shown to inhibit GSK-3β kinase activity. Although the peptide inhibited WT-GSK-3 kinase activity by 82% (Fig. 4c), it had very little effect on the activity of the Q89A and N95A mutants (Fig. 4c). This suggests that Gln89 and Asn95 interact with the pseudosubstrate and are important for its inhibitory function; the interaction can be direct (physical) or mediated by water molecules.

Molecular model of the GSK-3β–pseudosubstrate interactions

The evolutionary invariability of Arg4 and Arg6 together with the experimental data suggested that the interaction of the pseudosubstrate with the kinase is not random but rather specific. This encouraged us to dock the pseudosubstrate peptide into the active site of GSK-3β. The position of the pseudosubstrate peptide in our model is biased by the restraints imposed in the early stages of the minimization: Phosphorylated Ser9 was directed to bind in the vicinity of the phosphate binding pocket on the surface of GSK-3β, and Pro5 was restrained to reside near the P-loop. The first restraint was justified by ample experimental evidence that support binding of a phosphorylated group in the positively charged pocket formed by residues Arg96, Arg180, and Lys206 of the kinase. The second restraints rely on Pro5 being in the position of the potential phosphorylation site according to the SXXXS(p) motif. The last cycles of the intermittent molecular dynamics and energy minimization produced very similar structures for the N-terminal end of the docked pseudosubstrate peptide, residues Gly3–
Ala11. In contrast, the C-terminal end of the docked peptide including residues Ser12–Lys15 adopted somewhat different conformations after each minimization. The model of GSK-3β interaction with residues 3–11 of its pseudosubstrate peptide is presented in Fig. 5. Our model suggests that Arg6 and Arg4 are involved in direct interactions with GSK-3β. Arg6 forms hydrogen bonds with Gln89 and Asn95, and this is consistent with our previous study that showed the role of these sites in substrate recognition and binding. Arg4 is hydrogen bonded to Asp181, which is highly conserved among all protein kinases and accepts a proton from the hydroxyl group of the substrate. This may explain the inhibitory role of Arg4 as its interaction with Asp181 likely interferes with substrate binding.

Residues Thr7 and Thr8 are not in contact with the kinase. Thr7 forms a hydrogen bond with the carboxyl oxygen of Arg2, presumably stabilizing the binding conformation of the peptide. Thr8 is mostly exposed to the solvent, making only a loose contact with Val214. Loose contacts occur also between Phe10 and Ala11 and Phe93 of the kinase.

In summary, the results described here provide molecular details of the autoinhibition mechanism of GSK-3β. As previously demonstrated, phosphorylated Ser9 from the N-terminal region of the protein binds to a positively charged pocket. As shown here, the invariant Arg4 and Arg6 are required for this high-affinity binding of the N-terminal domain with the catalytic core. The interaction of the pseudosubstrate region with the catalytic core is facilitated at least in part by Gln89, Asn95, and Asp181.

**Experimental Procedures**

**Peptides and materials**

Peptides, including p9CREB, ILSRRPS(p)YR, pseudosubstrate peptide, and RPRTTS(p)FAES, were synthesized by Genemed Synthesis, Inc. (San Francisco, CA).

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**Fig. 4.** GSK-3β mutants Q89A and N95A are not inhibited by the pseudosubstrate. (a) HEK-293 cells were transiently transfected with plasmids coding for WT-GSK-3, Q89A, N95A, Q89/N95, or empty vector (control, C). Expression of GSK-3 proteins and their phosphorylation at Tyr216 were determined by Western blot analysis and are shown in the lower panel. In vitro kinase assays are shown in the upper panel. The proteins were subjected to in vitro kinase assays with p9CREB peptide substrate as described in Experimental Procedures. Reactions were spotted on p81 paper and counted for radioactivity. Results show phosphorylation of p9CREB and are presented as the percentage of WT-GSK-3 activity (set to 100% after subtracting protein kinase activity obtained from control cells expressing the empty vector). Results are the mean of five independent experiments ± SEM. *P < 0.01 for Q89A, N95A, and Q89/N95 mutant versus WT. (b) Cells expressing WT-GSK-3, Q89A, N95A, and S9A were treated with okadaic acid as described in Experimental Procedures. The proteins were immunoprecipitated from cell extracts and subjected to in vitro kinase assays with p9CREB peptide substrate in the presence of γ-ATP. Reactions were spotted on p81 paper and counted for radioactivity. Results show phosphorylation of p9CREB and are presented as the percentage of WT-GSK-3 activity (set to 100% after subtracting protein kinase activity obtained from control cells expressing the empty vector). Results are the mean of four independent experiments ± SEM. *P < 0.05 for WT treated cells versus nontreated cells (with the pseudosubstrate peptide). (c) The GSK-3 proteins were subjected to in vitro kinase assays as described in (a) except that in some samples, 2 mM of N-terminal peptide was added. Results show phosphorylation of p9CREB and are presented as the percentage of WT-GSK-3 activity as described. Results are the mean of three independent experiments ± SEM. Black bars indicate experiments without N-terminal peptide, and gray bars denote those with N-terminal peptide. *P < 0.05 for WT treated cells versus nontreated cells (with the pseudosubstrate peptide).
USA). GSK-3β antibody was from Transduction Laboratory (Lexington, KY, USA). Anti-phospho-GSK-3β (Y216) antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Anti-phospho-GSK-3 (S9) was from Cell Signaling Technology (Beverly, MA, USA). Radioactive materials were purchased from Amersham Biosciences (Piscataway, NJ, USA).

Plasmids and mutants

We previously described GSK-3β constructs in pCMV4 and pCDNA3 plasmids.23 These expression vectors were used as templates for mutagenesis of GSK-3β by the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) to replace Arg4, Pro5, Arg6, Ser9, Phe10, and Glu12 with alanine. Q89 and N95 mutants were previously described.22 The double mutant in which both Gln89 and Asn95 were mutated to alanine was generated by QuikChange Site-Directed Mutagenesis Kit as described. All constructs were sequenced to confirm the presence of mutations. The sequences of mutagenic oligonucleotides are available from the authors upon request.

In vitro translation of GSK-3 proteins

WT and mutant GSK-3 proteins encoded in pCDNA3 plasmids were expressed using the TNT transcription/translation kit (Promega, Madison, WI, USA) using T7 RNA polymerase. The reaction mixture contained DNA, rabbit reticulocyte lysate, RNA polymerase, and an amino acid mixture as outlined by the manufacturer. Following transcription and translation, equal amounts of proteins were diluted with 500 μL of buffer H [50 mM β-glycerophosphate, pH7.3, 1.5 mM ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid, 1 mM ethylenediaminetetraacetic acid, 2 mM DTT, 1 mM benzamidine, 25 μg/mL aprotinin, 25 μg/mL leupeptin, and 1 μg/mL pepstatin]. The proteins were immunoprecipitated with polyclonal antibody against GSK-3β in complex with Protein A Sepharose. The immunoprecipitates were washed twice with 0.5 M LiCl in 50 mM Tris, pH7.4, and with 50 mM Tris, pH7.4, and 1 mM DTT and were subjected to in vitro kinase assays.

Cells and transfections

HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. HEK-293 cells were transiently transfected with indicated constructs (5 μg each) using the calcium phosphate method as previously described.23 After 48 h, cells were treated with 200 μM okadaic acid (Sigma, St. Louis, MO, USA) for 1 h or left untreated. Cells were then lysed in ice-cold buffer G [20 mM Tris, pH7.5, 10 mM β-glycerophosphate, 10% glycerol, 1 mM ethylene glycol bis(β-aminoethyl ether)N,N'-tetraacetic acid, 1 mM ethylenediaminetetraacetic acid, 50 mM NaF, 5 mM tetrasodium diphosphate, 0.5 mM orthovanadate, 1 mM benzamidine, 10 μg/mL leupeptin, 5 μg/mL aprotinin, 1 μg/mL pepstatin, 500 nM microcystine LR, and 0.5% Triton X-100]. Cell extracts were centrifuged for 20 min at 12,000g. Supernatants were collected and equal amounts of proteins (20 μg) were boiled with SDS sample buffer and subjected to gel electrophoresis (10% polyacrylamide), transferred to nitrocellulose membranes, and immunoblotted with indicated antibodies.

In vitro GSK-3 kinase assays

GSK-3 proteins were immunoprecipitated from the translation mixture or from cell extracts with specific antibody. The immunoprecipitates were incubated with p9CREB substrate (100 μM) in a reaction mixture that contained 50 mM Tris/HCl, pH7.3, 10 mM magnesium acetate, 0.01% β-mercaptoethanol, and 32P[γ-ATP] (100 μM, 0.5 μCi/assay) for 20 min at 30 °C. Reactions were stopped with SDS sample buffer, spotted on p81

![Fig. 5. A docking model of the pseudosubstrate peptide interactions with GSK-3β. GSK-3β is depicted by the gray ribbon. Only the N-terminal part of the pseudosubstrate peptide (3-GRPRITSpA-11), whose structure did not vary significantly in the several minimizations, is shown as a golden ribbon. The side chains of interacting residues are shown with the oxygen atoms colored in red, nitrogen atoms in blue, and phosphor in green. Ser66 and Phe67 are shown to indicate the position of the P-loop. The binding position of ATP is indicated although this molecule was not included in the energy minimization.](image)
paper (Whatman, Kent, UK) washed with phosphoric acid, and counted for radioactivity. Similar experiments were performed to measure the activity of GSK-3 proteins expressed in HEK-293 cells. The activity of the endogenous GSK-3 that was determined in the cells transfected with the “empty” pCMV4 vector was subtracted from the activity values obtained for WT and mutants. In a different set of experiments, the reactions also included 2 mM of the N-terminal peptide RPRTTS(p)FAES.18 In vitro autophosphorylation assays were performed with 250 μM ATP in the absence of substrate. The reactions were incubated for 25 min at 30 °C. Reactions were boiled with SDS sample buffer, and the proteins were subjected to gel electrophoresis (10% polyacrylamide), transferred to nitrocellulose membranes, and immunoblotted with indicated antibodies.

Sequence analysis

The BLAST program24 was used to search for GSK-3 sequences in the National Center for Biotechnology Information sequence databases. The DIALIGN25 and MEME26 programs were used to create protein multiple sequence alignments. Sequence logos were created as described in Ref. [27]. A block multiple sequence alignment was created starting from described pseudo-substrate regions of GSK-3b.16,18 Comparing the block to other GSK-3 sequences identified more such regions, that were added to the block. The score cutoff for adding a region to the block for the next iteration was a calibrated block score of 1100, i.e., a ratio of 1.1 between the score of the region to the present block and the 99.5 percentile score of the block with unrelated sequences in a protein sequence database. Some known GSK-3 were not found to have a pseudo-substrate region (i.e., nematode, yeast, plant).

Molecular modeling

A model of a complex between GSK-3β catalytic domain and its pseudosubstrate region 13-amino-acid peptide 3-GRPRRTTS(p)FAESCK-15 was constructed by placing the extended peptide next to GSK-3β, surrounding both the protein and peptide by a layer of water molecules (7 Å thick) and energy minimizing the whole ensemble. We used the coordinates of GSK-3β in the Protein Data Bank28 entry 1h8f16 except that the conformation of Tyr216 was required that the Ser9(p) phosphorous atom resides at a distance of up to 5.0 Å from atoms Nζ of Lys205 and Cβ of Arg96 and Arg180. These restraints were rather general; hence, a “flat-bottomed” energy function was employed, which required that the Ser9(p) phosphorous atom resides at a distance of up to 5.0 Å from atoms Nζ of Lys205 and Cβ of Arg96 and Arg180. In this way, Ser9(p) was constrained to the vicinity of the positive binding pocket of GSK-3β but no specific hydrogen bond contacts were imposed. Similarly, general distance restraints were imposed on Pro5 to direct it to the vicinity of the GSK-3β P-loop. After several intermittent molecular dynamics and energy minimization cycles, only minor structural changes were observed and the computations were considered complete. All the restraints were released in the last energy minimization cycle. The computations were performed using the Discover-3 module of InsightIII (Accelrys Inc., San Diego, CA).

Statistics

Graphics and statistical analyses were performed using Origin Professional 6.0 software (OriginLab Corporation, Northampton, MA, USA). The significance of differences among experimental conditions was determined using the two-tailed Student’s t test. Data were deemed significant when P<0.05. Results were expressed as group mean with standard error of the mean (SEM). Mean values indicated in the text are of SEM<5%.

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References


