Fluorescence Studies on the Interaction of Inhibitor 2 and Okadaic Acid with the Catalytic Subunit of Type 1 Phosphoprotein Phosphatases†

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ABSTRACT: Phosphatase inhibitor 2 was mutagenized and expressed in *Escherichia coli* to produce a protein with a single cysteinyl residue at position 129. The newly introduced sulfhydryl group was labeled with a maleimide derivative of coumarin (CPM). The resulting fluorescent inhibitor 2 molecule (CPM-I2) retains biological activity and binds to the catalytic subunit of type 1 phosphatase (PP1-C) with a K_d similar to the K_i of native I2 (2–3 nM). Fluorescence anisotropy data indicate that kinase F_A (glycogen synthase kinase 3) does not dissociate the CPM-I2·PP1-C complex but rather causes a conformational change in the I2 molecule that is retained even after the CPM-I2 is displaced by an excess of native I2. The fluorescence data presented here also indicate that okadaic acid and I2 are competitive for binding to PP1-C, even after kinase F_A treatment of the CPM-I2·PP1-C complex.

The catalytic subunit of type 1 phosphatase is the natural and specific target for inhibitor 2 and, together with the catalytic subunit of type 2A phosphatase, the target of okadaic acid [cf. Cohen and Cohen (1989)]. The latter compound is produced by dinoflagellates and when concentrated in the digestive tracts of shellfish is a major cause of diarrhetic shellfish poisoning in humans (Yasumoto et al., 1985). Okadaic acid has been the focus of major interest since the first reports that this tumor promoter (Suganuma et al., 1988) is a specific inhibitor of type 1 and type 2A phosphatases both in vitro (Bialojan & Takai, 1988) and in vivo (Haystead et al., 1989).

The catalytic subunits of type 1 and type 2A phosphatases do not exist in free form in vivo (Cohen, 1989). The type 1 catalytic subunit (PP1-C),[1] the subject of this paper, is bound through a protein called the G subunit to glycogen in mammalian skeletal muscle and liver (Stralfors et al., 1985; DePaoli-Roach, 1989; Wera et al., 1991) and appears to be bound to other intracellular structures by related proteins [cf. Cohen (1989)]. Another population of PP1-C can be isolated from the cytosol of mammalian cells as a complex with inhibitor 2 (Yang et al., 1981; Ballou et al., 1983; Villa-Moruzzi et al., 1984; Tung & Cohen, 1984; Price et al., 1986; Vandenheede et al., 1989a). The heat-stable inhibitor 2 (I2) constitutes a modulator subunit for this inactive phosphatase and is required for the reversible activation–inactivation of the enzyme (Yang et al., 1981; Vandenheede et al., 1981). The I2·PP1-C complex is often referred to as the inactive ATP/Mg^{2+}-dependent phosphatase. A large body of literature exists on its activation by the protein kinase F_A [also known as glycogen synthase kinase 3; reviewed by Ballou and Fischer (1986) and Vandenheede et al. (1989b)].

Fluorescence techniques have been used to study the protein–protein interaction between PP1-C and I2 and the effect of okadaic acid (OA) on this interaction. Site-directed mutagenesis was employed to generate a mutant form of I2 which contains a cysteinyl residue in place of the serine at position 129. Native I2 does not contain cysteine (Holmes et al., 1986). Cys-129 in the mutant I2 was derivatized by reaction with a maleimide derivative of coumarin, CPM. Neither substitution of cysteine for serine nor derivatization greatly affected the I2 inhibition of PP1-C activity. The fluorescence data presented here indicate that the fluorescent I2 binds to PP1-C with a K_d which is similar to the K_i of native I2 and that the protein kinase F_A in the presence of ATP/Mg^{2+} affects the inhibitor within the I2·PP1-C complex. OA and I2 each competitively inhibit binding of the other to PP1-C.

Materials and Methods

Materials

Phosphorylase *b*, phosphorylase kinase, and histones IIA were purchased from Sigma, St. Louis, MO. The coumarin derivative 3-(4-maleimidophenyl)-4-methyl-7-(diethylamino)coumarin (CPM) was from Molecular Probes, Eugene, OR. [γ-^{32}P]ATP was obtained from NEN Research Products, Boston, MA. All chemicals were of reagent grade. Okadaic acid was a kind gift from Dr. Francis Schmitz, Oklahoma

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[1] Abbreviations: Pipes, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; LB broth, Luria broth; CPM, 3-(4-maleimidophenyl)-4-methyl-7-(diethylamino)coumarin; native I2, phosphatase inhibitor 2 isolated from rabbit skeletal muscle; I2Cys129, mutant I2 containing a cysteine at position 129; CPM-I2, I2Cys129 labeled with CPM at position 129; PP1-C, catalytic subunit of protein phosphatase type 1; PP2A, type 2A protein phosphatase; kinase F_A, protein that activates the I2·PP1-C complex in an ATP/Mg^{2+}-dependent reaction; OA, okadaic acid.

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Methods

Isolation of Proteins. The catalytic subunit of the type 1 phosphatase (PP1-C) was isolated from rabbit skeletal muscle essentially as described by Tung et al. (1984), except that a heparin–Sepharose concentration step was included in the procedure: the catalytic subunit was adsorbed in 20 mM Tris-HCl (pH 8.0)/1 mM DTT containing 50 mM NaCl and eluted with 0.4 M NaCl in the same solution. Its specific activity was between 5000 and 10000 units/mg (1 unit equals 1 nmol of phosphate released from the substrate per minute).

Native I2 was isolated from rabbit skeletal muscle as reported by Vandenheede et al. (1989a). Native type 2A phosphatase was purified from rabbit reticulocytes by the procedure of Chen et al. (1989). Antibodies against I2 were prepared as previously reported (Roach et al., 1985). Protein kinase F_A was partially purified from the rabbit reticulocyte postribosomal supernatant fraction by chromatography on DEAE-cellulose and CM-Sephadex as described in Wollny et al. (1984). Casein kinase II was also isolated from the rabbit reticulocyte postribosomal supernatant fraction as indicated previously (Rose et al., 1987).

Enzyme Assays. The kinase F_A and ATP/Mg^{2+}-dependent assay of the I2·PP1-C complex is described in Wollney et al. (1984). Phosphorylase *a* was the substrate in this assay which was carried out in a volume of 30 μL containing 40 mM Pipes–KOH (pH 7.0), 2.5 mM dithioerythritol, 5 mM caffeine, and 0.5 mg/mL bovine serum albumin with the amounts of PP1-C and substrate given in the figure or table legends. Release of phosphate was quantitated by the method described in Wollny et al. (1984). PP1-C activity was determined usually after a 10-min incubation at 35 °C under the same conditions omitting the preincubation step with kinase F_A and ATP/Mg^{2+}. Phosphorylase *a* was prepared from phosphorylase *b* by incubation with phosphorylase kinase and [γ-^{32}P]ATP as previously reported (Krebs et al., 1958).

Cloning of Phosphatase Inhibitor 2 cDNA. Rabbit skeletal muscle I2 cDNAs were isolated from libraries constructed in λgt11 bacteriophage (Zhang et al., 1989). Overlapping cDNA clones provided an open reading frame of 612 nucleotides corresponding to 204 amino acids (Park and DePaoli-Roach, unpublished results). Analysis of DNA sequences demonstrated that the deduced primary structure of the encoded polypeptide is identical to that reported from protein sequencing (Holmes et al., 1986, 1987).

Site-Directed Mutagenesis. Mutant I2 was generated in which Cys replaced the Ser at position 129 by in vitro site-directed mutagenesis. A 27-mer mutant oligonucleotide, in which the serine-129 codon TCA was substituted with the cysteine codon TGT, was synthesized and annealed to single-stranded M13 containing the 395 bp I2 *Bam*HI–*Hin*dIII cDNA fragment. Mutagenesis reactions were carried out using the Amersham oligonucleotide-directed mutagenesis system 2 according to the manufacturer's instructions (Taylor et al., 1985). The mutagenized sequence was confirmed by single-stranded DNA sequencing (Sanger et al., 1977). The replicative form of M13 DNA was prepared; the *Bam*HI–*Hin*dIII fragment containing the mutation was excised and used to replace the corresponding wild-type fragment in I2·pTRCpUCII vector (Park and DePaoli-Roach, unpublished results).

Expression of I2Cys129. I2Cys129·pTRCpUCII was introduced into *Escherichia coli* 222. Colonies were grown in 10 mL of LB broth plus 0.1 mg/mL ampicillin overnight at 37 °C. Cells were collected by centrifugation at 7500*g* for 10 min and resuspended in 10 volumes of homogenization buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% β-mercaptoethanol, 0.5 mM PMSF, 0.1 mM TLCK, 2 mM benzamidine, and 10 μg/mL leupeptin). Extracts were prepared by sonication (4 × 30 s) followed by a 5-min treatment at 100 °C and centrifugation for 5 min at 4 °C in a microfuge. The supernatant was analyzed for type 1 phosphatase inhibitory activity and for the presence of a 31-kDa polypeptide on SDS–PAGE stained with Coomassie blue. For large-scale purification, *E. coli* 222 was freshly transfected with the mutant expression construct and grown in 5 mL of LB broth plus ampicillin until the absorbance at 595 nm was 0.4. A half-liter of LB medium plus ampicillin was then inoculated for overnight growth. Cells were harvested and resuspended in 8 volumes of homogenization buffer. After one cycle of freezing–thawing, lysozyme was added to a final concentration of 30 μg/mL, and the preparation was incubated for 20 min on ice. After a second cycle of freezing–thawing, the suspension was sonicated (4 × 30 s). The cell lysate was treated at 100 °C for 15 min and then centrifuged for 20 min at 13000*g*. The supernatant was used to purify I2Cys129 by the method previously described (DePaoli-Roach, 1984).

I2Cys129 was purified close to homogeneity as judged by Coomassie blue staining of an SDS–polyacrylamide gel (data not shown). The specific activity of I2Cys129 was 2.3×10^6 units/mg of protein, which is very similar to that of native I2. One unit of I2 is the amount of polypeptide which causes 50% inhibition of 4 milliunits of PP1-C.

Fluorescence Labeling of Mutant I2. Initial efforts to label native I2 on the lysyl ε-amino groups resulted in inactivation of the protein. Because of this, the I2Cys129 mutant was developed by inserting cysteine into a position which had been shown not to be important for PP1 inhibition (Park and DePaoli-Roach, unpublished results).

I2Cys129 was dialyzed against 20 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. For coumarin labeling, 200 μL of a 5 mM CPM solution in dimethylformamide (DMF) was added to an equal volume of I2Cys129, and the mixture was incubated at 37 °C for 30 min. The majority of unreacted CPM was then precipitated by adding 100 μL of water, chilling the mixture on ice for 15 min, and centrifuging the sample at 15000*g* for 10 min. The remainder of the unreacted CPM was then removed by gel filtration on Sephadex G-50.

The molar ratio of CPM incorporated into I2Cys129 was estimated to be approximately 1:1. The concentration of the coumarin-labeled I2Cys129 (CPM-I2) was determined by the absorbance at 388 nm (extinction coefficient of CPM = 30000). A single fluorescent protein band was observed by SDS–polyacrylamide gel electrophoresis on 15% gels with no fluorescence appearing at the dye front.

Fluorescence Measurements. Samples for fluorescence measurements were prepared directly in cuvettes containing 600 μL of 20 mM Pipes–KOH (pH 7.0) and additions indicated for the individual experiments. Binding reached equilibrium rapidly (<5 min) prior to fluorescence measurements.

A photon-counting spectrofluorometer, Model 8000 from SLM–Aminco Instruments Inc. (Urbana, IL), was used to carry out steady-state fluorescence measurements as described previously (Rychlik et al., 1983). When spectra were taken, data were accumulated at 1-nm intervals with a scanning rate of 0.5 s per wavelength increment. Spectra were automatically corrected for the wavelength dependence of photomultiplier sensitivity. All fluorescence measurements were made at 20 °C and at an absorbance of less than 0.1 at the wavelength of excitation (385 nm). Steady-state fluorescence anisotropy

measurements were made as described (Odom et al., 1984).

Calculation of the K_i for Okadaic Acid. In order to examine the effect kinase F_A and native I2 have on the binding of OA to PP1-C, the inhibition of phosphatase activity by OA was measured for PP1-C (in the presence and absence of kinase F_A) and for the kinase F_A reactivated I2·PP1-C complex. The release of [^{32}P]P_i from [^{32}P]phosphorylase *a* was measured, and the K_i for OA was calculated. The latter value was used as an index for the ability of OA to bind to the phosphatase. We assume K_i to equal K_d of the OA·PP1-C complex; i.e., we assume a single binding site for OA on PP1-C that is responsible for inhibition.

The phosphatase activity in the absence of OA is defined as 1 and is equivalent to the initial activity (v_0). The activity measured after the addition of a given amount of OA (v_i) is then used to give the fraction of activity remaining (v_i/v_0). The fraction of activity inhibited is calculated by $1 - v_i/v_0$ which is equivalent to $(v_0 - v_i)/v_0$. v_0 follows Michaelis–Menten kinetics. If OA binding is noncompetitive with [^{32}P]-phosphorylase *a* binding (Bialojan & Takai, 1988), then

$$v_i = \frac{V_{max}[S]}{K_m(1 + [OA]/K_i) + [S](1 + [OA]/K_i)}$$

where [S] is the concentration of substrate ([^{32}P]phosphorylase *a*), K_m is the Michaelis constant, and V_{max} is equal to v_0 at saturating substrate concentration; [OA] is the concentration of OA, and K_i is the inhibition constant of OA.

$$\frac{v_i}{v_0} = \frac{K_m + [S]}{K_m(1 + [OA]/K_i) + [S](1 + [OA]/K_i)}$$

which can be rearranged and inverted to give

$$\frac{v_0}{v_0 - v_i} = \frac{1 + [OA]/K_i}{[OA]/K_i} = 1 + K_i(1/[OA])$$

According to this equation, a plot of 1/(the fraction of phosphatase activity inhibited) versus 1/[OA] should be linear with an intercept on the ordinate at a value equal to 1/(the fraction of activity inhibited at an infinite OA concentration) (which should be 1), and an intercept on the abscissa which equals $-1/K_i$ for OA.

Results

Inhibitory Activity of Native I2, I2Cys129, and CPM-I2 with PP1-C in Vitro. A mutant form of I2 containing a cysteinyl residue substituted for the serine at position 129 was fluorescently labeled on the newly introduced sulfhydryl group with a maleimide derivative of coumarin (CPM). Under the assay conditions used, native I2 and I2Cys129 gave 50% inhibition of PP1-C phosphatase activity at about 2 nM, while the CPM-labeled inhibitor (CPM-I2) gave 50% inhibition at about 2.5 nM (Figure 1). These data indicate that neither the substitution at position 129 nor the covalent attachment of a coumarin probe at this position has a marked effect on the inhibitory activity of I2.

Fluorescence Properties of CPM-I2 before and after Binding to PP1-C. Three fluorescence parameters were determined for CPM-I2 in solution. Fluorescence intensity, fluorescence anisotropy, and emission maximum were measured before and after the addition of various proteins (Table I). Fluorescence from coumarin is sensitive to the local environment of the probe. An increase in the fluorescence quantum yield and a shift in the emission spectrum toward the blue generally are observed in solvents of greater hydrophobicity. The fluorescence emission maximum (476 nm) of the labeled I2 was shifted to the blue by 4 and 7 nm, respectively, when PP1-C- or I2-specific polyclonal antibodies were added. This shift in the emission spectrum reflects the interaction of these proteins with the labeled I2. Increases in fluorescence intensity of 52% and 25%, respectively, also were observed when an excess of PP1-C or antibodies specific for I2 was added to the solution. These results suggest that an increase in the hydrophobicity around the probe is associated with binding of CPM-I2 to these proteins. No similar effect was seen with nonimmune serum or the other proteins tested which included kinase F_A and PP2A. Trypsinolysis of CPM-I2 caused a 20% decrease in fluorescence intensity, apparently by exposing the fluorophore to the more aqueous environment of the surrounding solution.

Fluorescence anisotropy was used as a third means to monitor CPM-I2 interaction with PP1-C. Depolarization of fluorescence occurs if the orientation of the probe relative to the excitation beam changes while the probe is in the excited state. Thus, changes in fluorescence polarization may be used to monitor changes in the mobility of the probe and movement in solution of the macromolecule to which the probe is attached. Binding of CPM-I2 to PP1-C causes an increase in mass from about 23 000 daltons for the inhibitor free in solution to

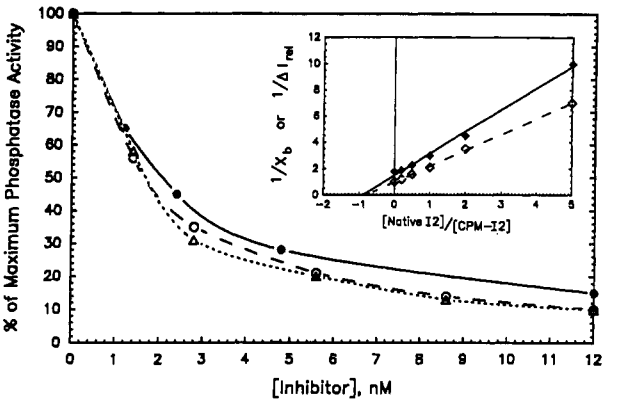

FIGURE 1: Inhibition of PP1-C activity by native I2, I2Cys129, and CPM-I2. Enzymatic activity of PP1-C (0.2 nM) was determined in the absence and presence of native I2 (△), I2Cys129 (○), or CPM-I2 (●). The concentrations of the inhibitors are indicated on the abscissa. 100% activity equals 21 pmol of phosphate released by PP1-C from the substrate (phosphorylase *a*, 232 pmol). Inset: Competition between native I2 and CPM-I2. The fluorescently labeled I2 (20 nM) and native I2 were mixed to give various molar ratios; then PP1-C (21 nM) was added, and the mixtures were incubated for 5 min at 20 °C. The anisotropy and fluorescence intensity of the CPM-I2 were then determined. The change in anisotropy was used in eq 1 to determine the fraction of CPM-I2 bound (X_b). $1/X_b$ (◇) and the reciprocal of the change in fluorescence intensity (◆) were then plotted versus the molar ratio of unbound native inhibitor to unbound fluorescent inhibitor which was calculated from X_b.

Table I: Changes in Fluorescence Parameters upon Specific Binding of Proteins to CPM-I2Cys129[a]

additions	emission maximum (nm)	relative intensity	anisotropy
none	476	1.00	0.234
PP1-C	472	1.52	0.302
PP2A	475	1.09	0.240
BSA	475	1.10	0.241
kinase F_A	475	1.10	0.245
immune serum	469	1.25	0.326
nonimmune serum	477	1.00	0.240
trypsin	477	0.80	0.151

[a] Each cuvette contained CPM-I2 (20 nM) in 20 mM Pipes–KOH (pH 7.0) in a total volume of 600 μL, and where indicated the following additions: PP1–C, 12 μg; BSA, 20 μg; PP2A, 2 μg; kinase F_A, 6 μg; trypsin, 0.5 μg. Immune and nonimmune sera (1 μL of each) were also tested. After the additions, the cuvette was incubated for 10 min at 20 °C, before the fluorescence measurement was carried out.

about 60000 daltons for a 1:1 inhibitor–phosphatase complex. This increase in the mass would be expected to significantly increase the time required for tumbling of CPM-I2 in solution and thus to increase fluorescence anisotropy. The anisotropy for free CPM-I2 was determined to be about 0.234; this value increased to 0.302 in the presence of a large excess of PP1-C (Table I). Of the other proteins tested, only the polyclonal antibodies raised against I2 caused an increase in fluorescence anisotropy. Trypsinolysis of CPM-I2 caused a decrease in fluorescence anisotropy (Table I). The fluorescence anisotropy of free coumarin-labeled cysteine was about 0.065 (data not shown).

Dissociation Constant and Competition with Native I2. To estimate the affinity of CPM-I2 for PP1-C, the fluorescent inhibitor was incubated with increasing concentrations of PP1-C, and the change in fluorescence anisotropy was monitored. The CPM-I2 concentration was held constant at 15 nM in these experiments in order to maintain levels of fluorescence which could be accurately measured. Changes in anisotropy were corrected for changes in fluorescence intensity by the equation:

$$\frac{\Delta A_{obs}}{(q_b/q_f)\Delta A_{max} - (q_b/q_f - 1)\Delta A_{obs}} = X_b \qquad (1)$$

where X_b is the fraction of CPM-I2 which is bound, ΔA_{max} is the maximum possible change in fluorescence anisotropy (estimated from a plot of PP1-C concentration versus anisotropy extrapolated to infinite PP1-C concentration), ΔA_{obs} is the observed change in anisotropy, and q_b/q_f is the ratio of fluorescence intensities of bound and free CPM-I2 [Ellerton & Isenberg, 1969; recently applied by Odom et al. (1990)]. This equation corrects for the increase in fluorescence intensity that occurs upon CPM-I2 binding to PP1-C. Without correction, the observed anisotropy is weighted toward that of the bound form of CPM-I2. The resulting plot (not shown) indicates that the CPM-I2-PP1-C complex has an apparent K_d of about 2.5 nM. This value is very close to the concentration of fluorescent inhibitor required for 50% inhibition of PP1-C enzyme activity (see Figure 1). Because the CPM-I2 concentration necessary for the fluorescence measurements is significantly higher than the concentration required for 50% inhibition of PP1-C enzyme activity (see Figure 1), the apparent K_d value determined by this method is subjected to the error inherent in determining the relatively small proportion of unbound CPM-I2.

Binding of CPM-I2 to PP1-C also was determined by competition with native I2. Fluorescence anisotropy and the intensity of CPM-I2 with PP1-C were measured in the presence of increasing amounts of native I2. The fraction of bound fluorescent inhibitor (X_b) was calculated from the observed changes in anisotropy as described above (see eq 1). The results are given as the inset in Figure 1, where $1/X_b$ and $1/\Delta I_{rel}$ are plotted against the ratio of [native I2]/[CPM-I2]. The ratio of the K_d values, $-(K_{d,native\ I2}/K_{d,CPM\text{-}I2})$, is given by the intercept on the abscissa. In agreement with the enzyme inhibition data shown in the main part of Figure 1, the K_d of the native I2·PP1-C complex is approximately the same (30% lower) as that with the fluorescent inhibitor.

Effect of Kinase F_A and ATP/Mg^{2+} on the Fluorescence of CPM-I2 Bound to PP1-C. Protein kinase F_A (also known as glycogen synthase kinase 3) has been reported to activate the inactive I2-PP1-C complex in an ATP/Mg^{2+}-dependent reaction, and a number of models have been proposed to explain the mechanism by which this activation of PP1-C is brought about [reviewed by Ballou and Fischer (1986) and by Vandenheede et al. (1989b)]. Transient phosphorylation of I2 in the complex with PP1-C by kinase F_A appears to be involved (Hemmings et al., 1982; Ballou et al., 1983). This phosphorylation is synergistically increased by casein kinase II (DePaoli-Roach, 1984). Free CPM-I2 was phosphorylated by kinase F_A to a similar extent as native I2, about 0.15 mol of phosphate incorporated per mole of inhibitor. Low phosphorylation of I2 by kinase F_A has been observed and discussed previously (Hemmings et al., 1982; Ballou et al., 1983; DePaoli-Roach, 1984).

Table II: F_A Does Not Dissociate the I2·PP1-C Complex

(A) Fluorescence Measurements[a] additions	anisotropy
(1) CPM-I2	0.234
(2) plus PP1-C	0.292
(3) plus ATP/Mg^{2+}	0.293
(4) plus F_A	0.309

(B) Enzymatic Activity[b] additions	phosphate released (pmol)
(1) PP1-C	57.1
(2) PP1-C, I2	12.7
(3) PP1-C, I2, ATP/Mg^{2+}	5.4
(4) PP1-C, I2, F_A	16.0
(5) PP1-C, I2, F_A, ATP/Mg^{2+}	42.0

[a] Fluorescence measurements were carried out as described under Methods. These were started with 15 nM CPM-I2; then PP1-C was added to give 15 nM. After addition of ATP and Mg^{2+} (0.2 and 1 mM, respectively), kinase F_A was added (about 6 μg of a partially purified preparation). After each addition, the cuvette was incubated for 5 min at 37 °C before measurement of anisotropy. [b] Assays for enzymatic activity were done in a total volume of 30 μL under conditions outlined under Methods using [^{32}P]phosphorylase *a* (250 pmol) as substrate. PP1-C (1.2 nM) was present in all tubes and I2Cys129 (16.2 nM) in tubes 2–5. Samples 3–5 additionally contained the components listed: ATP and Mg^{2+}, 0.2 and 1 mM, respectively; kinase F_A, 0.77 μg.

Fluorescence anisotropy confirmed that the PP1-C·I2 complex remains associated following activation by kinase F_A [cf. Ballou and Fischer (1986)]. Dissociation of the CPM-I2·PP1-C complex would be expected to result in a decrease in fluorescence anisotropy ultimately to the level seen with the free form of CPM-I2. The addition of an equimolar amount of PP1-C to CPM-I2 caused the expected increase in the anisotropy from 0.234 to 0.292 (Table II). A small but significant additional increase in anisotropy to 0.309 was seen when kinase F_A was added to the cuvette containing the CPM-I2·PP1-C complex and ATP/Mg^{2+} (Table II). Little or no change in anisotropy was observed in the presence of only ATP/Mg^{2+} or in the absence of PP1-C with kinase F_A present. Addition of PP1-C to the latter reaction mixture caused the anisotropy to increase to the value obtained when kinase F_A and ATP/Mg^{2+} were added to the preformed CPM-I2·PP1-C complex (data not shown). The results indicate that under the assay conditions used, incubation in the presence of kinase F_A and ATP/Mg^{2+} does not cause dissociation of the I2·PP1-C complex; rather, it leads to a small increase in anisotropy. This increase in anisotropy appears to be associated with a change in the conformation or state of the I2 molecule as indicated by the results of the experiments described below.

The affinity of CPM-I2 for PP1-C in the presence of kinase F_A and ATP/Mg^{2+} was evaluated from fluorescence anisotropy The dissociation constant of the CPM-I2·PP1-C complex was measured by holding the inhibitor concentration constant at 15 nM (in the presence or absence of kinase F_A plus ATP/Mg^{2+}) and adding increasing concentrations of PP1-C. The resulting changes in anisotropy were used to calculate the

fraction of CPM-I2 bound (X_b in eq 1). This value was then used to determine the concentration of free PP1-C. A double-reciprocal plot (not shown) of X_b vs $[PP1\text{-}C]_{free}$ evaluates the K_d of the complex in the presence and absence of kinase F_A and ATP/Mg^{2+}. The x intercept gives the reciprocal of the free PP1-C concentration required for 50% CPM-I2 binding (apparent K_d). As mentioned above, the CPM-I2 concentration required for these fluorescence experiments is significantly greater than K_d, thus potentially limiting the accuracy of the determination. However, the results indicate that kinase F_A causes an increase in K_d of the CPM-I2·PP1-C complex from 2.0 to 4.0 nM. This increase in K_d is associated with the increase in fluorescence anisotropy noted above (Table II). The results presented below suggest that this is the result of an effect of kinase F_A on CPM-I2 in the complex with PP1-C.

Native I2 was used as a competitive inhibitor of CPM-I2 for formation of the CPM-I2·PP1-C complex in the absence or presence of kinase F_A and ATP/Mg^{2+}. The experiments are analogous to those described above for determination of the relative K_d values of the two forms of I2 (see Figure 1, inset). The anisotropy of CPM-I2 fluorescence was higher in the presence of kinase F_A plus ATP/Mg^{2+} at all concentrations of native I2 that were tested (panel A of Figure 2, closed circles). A plot of $1/X_b$ versus the ratio of native I2 to CPM-I2 gives the ratio of the K_d values for native I2 and CPM-I2 as the intercept on the abscissa (panel B of Figure 2). The ratios are indistinguishable in the presence or absence of kinase F_A even though the K_d values themselves are changed, thus indicating that kinase F_A has a similar effect on native I2 and CPM-I2.

From the results presented in Figure 1, inset, it was anticipated that CPM-I2 would be largely displaced from the PP1-C complex at higher concentrations of native I2 and that this would be reflected in a drop in anisotropy to levels near those observed for free CPM-I2. This result was obtained in the absence of kinase F_A plus ATP/Mg^{2+} (panel A of Figure 2, open circles). However, as indicated above, the kinase caused an *increase* in anisotropy in the presence or absence of added native I2 even though the K_d for the CPM-I2·PP1-C complex had increased. This unanticipated result suggests kinase F_A plus ATP/Mg^{2+} causes a change in the state or conformation of CPM-I2 in the CPM-I2·PP1-C complex and that the change was retained in the CPM-I2 even after it was displaced from PP1-C by the native inhibitor. This finding was further explored by the experiments described in Figure 3 in which incubation of CPM-I2 with kinase F_A plus ATP/Mg^{2+} was carried out with a limiting amount of PP1-C. The ratio of PP1-C/CPM-I2 that was used was about 0.1 rather than about 1.0 as for the experiments in Table II. Upon the addition of PP1-C, a small but rapid increase in anisotropy was observed that appears to reflect the formation of the CPM-I2·PP1-C complex. It was followed by a relatively slow increase in anisotropy which after 30 min approached the value of 0.275 (Figure 3A). A similar value was reached in the experiments of Figure 2 (panel A, closed symbols) which were carried out in the presence of higher concentrations of native I2. The addition of EDTA to chelate Mg^{2+} in the reaction mixture (thereby blocking kinase F_A activity) was associated with a slow decline in anisotropy (Figure 3, panel B). Addition of excess PP1-C at any point in the sequence caused an increase in anisotropy to the level anticipated for formation of a CPM-I2·PP1-C complex (Figure 3, panel C). Our interpretation of these results is that the kinase F_A causes a change in CPM-I2 in the CPM-I2·PP1-C complex that is reflected by increased anisotropy even after the CPM-I2 is dissociated from PP1-C but that the modified I2 reverts slowly to its native state or conformation.

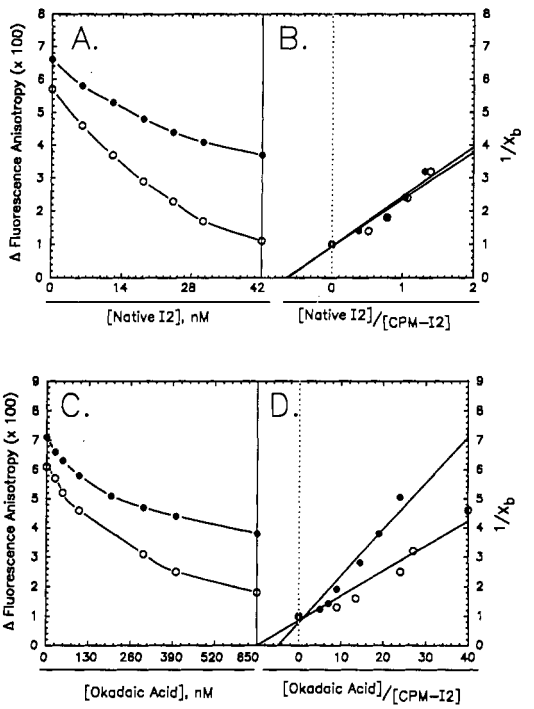

FIGURE 2: Displacement of CPM-I2 from PP1-C by native I2 (A, B) or okadaic acid (C, D) in the absence and presence of kinase F_A and ATP/Mg^{2+}. In (A), the change in fluorescence anisotropy for 15 nM CPM-I2 upon binding to a slight excess (17 nM) of PP1-C was determined in the presence (closed circles) and absence (open circles) of 6 μg of kinase F_A and ATP/Mg^{2+}. In each case, bound CPM-I2 was then competitively displaced from PP1-C by adding increasing amounts of native I2 and incubating the sample 10 min at 20 °C. The change in anisotropy (in the absence of kinase F_A) was then used to determine the bound CPM-I2 concentration according to eq 1. From this, the concentrations of unbound CPM-I2 and native I2 were determined, and the ratio of unbound native I2 and CPM-I2 was plotted against $1/X_b$ (panel B, open symbols). A lower limit for the anisotropy of CPM-I2 released from PP1-C in the presence of kinase F_A and ATP/Mg^{2+} was approximated by adding a large excess of native I2 (100 nM). This lower anisotropy limit (0.275) was then used to calculate X_b for CPM-I2 as it is released from PP1-C in the presence of kinase F_A. In this way, it is possible to calculate the concentrations of unbound native I2 and CPM-I2 in the presence of kinase F_A so that the ratio of these two values can be plotted against $1/X_b$ (panel B, closed symbols). The intercept on the abscissa in (B) gives the negative value of the ratio of the K_d of native I2 to the K_d of CPM-I2. In (C), CPM-I2 (20 nM) was bound to PP1-C (22 nM) in the presence (closed circles) and absence (open circles) of kinase F_A and ATP/Mg^{2+} exactly as described for (A). Increasing amounts of OA were added, and the subsequent decrease in fluorescence anisotropy was measured. In (D), the anisotropy change in the absence of kinase F_A (open symbols) was used to calculate the concentrations of unbound OA and CPM-I2 for use in a plot of $1/X_b$ versus the ratio of unbound OA to unbound CPM-I2 as in panel B. The lower limit of the anisotropy of released CPM-I2 (0.275) in the presence of kinase F_A was approximated by increasing the OA concentration to 5 μM. Having determined a lower limit for the released CPM-I2, X_b was calculated and used to determine the concentrations of unbound OA and unbound CPM-I2. The ratio of these values was plotted against $1/X_b$ (panel B, closed symbols). The intercepts on the abscissa give the negative ratios of the K_d values of OA to CPM-I2 in the presence (closed symbols) and absence (open symbols) of kinase F_A and ATP/Mg^{2+}.

Okadaic Acid. Recently Cohen and co-workers (McKintosh et al., 1990) presented indirect evidence indicating that I2 might prevent binding of okadaic acid (OA) to PP1-C. In an attempt to clarify the relationship between these two phosphatase inhibitors, we used fluorescence techniques to test the

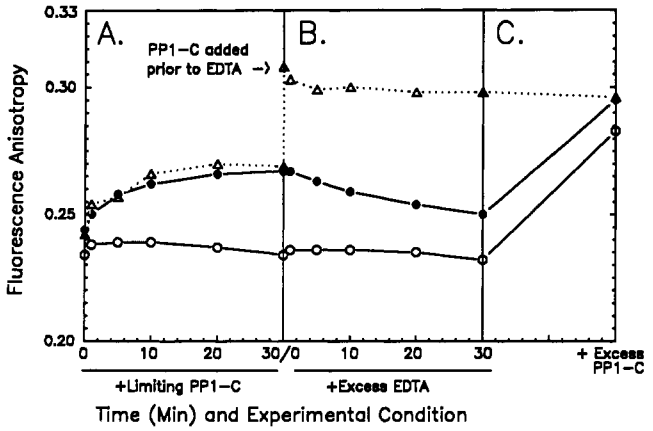

FIGURE 3: Effect of kinase F_A on the state or conformation of I2. CPM-I2 (30 nM) was incubated in the presence (●, △) or absence (○) of kinase F_A (5 μg) and ATP/Mg^{2+}. In panel A, a limiting concentration of PP1-C (3 nM) was added to each sample, and the time-dependent change in fluorescence anisotropy was monitored. After 30 min at 20 °C, in one of the mixtures containing kinase F_A the PP1-C (△) concentration was increased to 40 nM and the anisotropy measured. All the samples were then made 8 mM in EDTA to remove essentially all the available Mg^{2+} and thus render the kinase F_A inactive (panel B). Little or no change in the anisotropy of CPM-I2 in the sample lacking kinase F_A (○) was seen, but a marked, though slow, decrease in the anisotropy of the CPM-I2 in the presence of kinase F_A (●) was observed. The anisotropy of the CPM-I2 complexed with excess PP1-C in the presence of kinase F_A decreased at a somewhat faster rate (△) upon EDTA addition. To show that over the time of the experiment CPM-I2 had not been inactivated, an excess of PP1-C (40 nM) was added to the samples which still contained limiting PP1-C concentrations (○, ●), and then their anisotropy was measured (panel C).

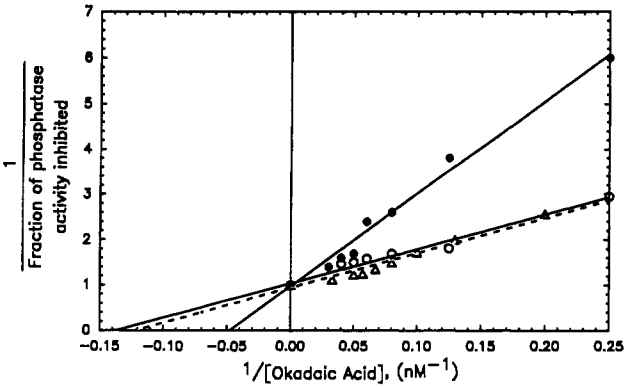

FIGURE 4: Inhibition by okadaic acid of PP1-C activity in the presence and absence of kinase F_A and of kinase F_A reactivated I2·PP1-C activity. A double-reciprocal plot of OA concentration versus the fraction of inhibited phosphatase activity is shown for PP1-C (open circles), in the presence of kinase F_A and ATP/Mg^{2+} (open triangles), and I2·PP1-C which has been reactivated by kinase F_A and ATP/Mg^{2+} (closed circles). For the latter experiment, 0.2 nM PP1-C was incubated 5 min at 37 °C with 8 nM native I2. About 0.5 μg of protein of a kinase F_A preparation was then added, and the mixture was made 1 mM in Mg^{2+} and 0.2 mM in ATP and incubated 10 min at 37 °C. Increasing concentrations of okadaic acid were then added and the reaction mixtures incubated 3 min at 37 °C. ^{32}P-Labeled phosphorylase *a* (240 pmol) and caffeine (3 mM final concentration) were added, and [^{32}P]phosphate released from the phosphorylase *a* was determined after 10 min at 37 °C. For the original PP1-C activity, the enzyme (0.2 nM) was incubated with increasing OA concentrations for 3 min at 37 °C, then caffeine and phosphorylase *a* were added, and [^{32}P]phosphate release was measured after 10 min at 37 °C.

effect of OA on formation of the CPM-I2·PP1-C complex. PP1-C was preincubated with CPM-I2 in the presence or absence of kinase F_A and ATP/Mg^{2+}; then OA at the indicated final concentrations was added to these samples. The data presented in panel C of Figure 2 indicate that OA causes a decrease in fluorescence anisotropy from CPM-I2 that is very similar to that observed with excess native I2 in the absence and presence of kinase F_A and ATP/Mg^{2+}. These data indicate that OA interferes with the formation or stability of the CPM-I2·PP1-C complex. In a second set of experiments, PP1-C was incubated first with an excess of OA to form a PP1-C·OA complex and then CPM-I2 was added. Fluorescence anisotropy was measured after 10 min of additional incubation. The results (not presented) were similar to those shown in panel C of Figure 2 (open symbols), indicating that an equilibrium had been established for competitive binding of OA and I2 to PP1-C.

The fraction of CPM-I2 in the PP1-C·CPM-I2 complex (X_b) was then calculated from the data given in Figure 2C, and plotted for the OA/CPM-I2 ratio (Figure 2D). The ratios of the K_d values for PP1-C complexed with the two inhibitors in the presence and absence of kinase F_A are given as intercepts on the abscissa. These are about 5 and 10, respectively. If the K_d values for CPM-I2 in the presence and absence of kinase F_A are taken as 4 and 2 nM, respectively, then the K_d for OA is 20 nM either in the presence or in the absence of kinase F_A. This result indicates that kinase F_A does not detectably affect the OA·PP1-C interaction and is consistent with the conclusion that its direct effect is on I2.

For comparison, the effect of OA on the phosphatase activity of PP1-C was determined, and the results are shown in Figure 4. The K_i for OA is 7 nM with PP1-C only or with PP1-C after incubation with kinase F_A plus ATP/Mg^{2+} in the absence of I2 (open symbols). The corresponding K_i for I2·PP1-C reactivated by kinase F_A was 22 nM. The K_i value for OA is in reasonable agreement with the K_d value of 20 nM that was determined by fluorescence measurements (Figure 2C,D). The fact that the K_i of OA for the reactivated form of I2·PP1-C is greater than for PP1-C alone may indicate that both types of determinations reflect the same basic competitive phenomenon.

This result is consistent with the reported IC_{50} value of OA for PP1-C of 10–15 nM (Cohen et al., 1989). IC_{50} values in excess of 100 nM have been reported for OA inhibition of type 1 phosphatases (Bialojan & Takai, 1988; Ishihara et al., 1989). The basis for the differences between these latter values and those reported here is not known but may be related to the association of PP1-C with other proteins in the enzyme preparations that were used.

Discussion

Recently, Csortos et al. (1990) determined a dissociation constant of 4.8 μM for the complex formed between I2 and a fluorescently labeled derivative of PP1-C. The covalent labeling (to about 90%) of PP1-C with a pyrene maleimide resulted in 90% reduction in the enzymatic activity and in binding of a substrate analogue, thiophosphorylase α. Here we report the results of a study involving fluorescence in which an engineered mutant form of I2, containing a cysteine in place of serine-129, was covalently labeled with a maleimide derivative of coumarin. The complex formed between CPM-I2 and PP1-C had a dissociation constant of 2.5 nM. This value is similar to that for native I2, 2.0 nM, calculated from experiments in which competition between CPM-I2 and native I2 was measured. These values are in good agreement with K_i values determined by measuring the phosphatase activity of PP1-C in the presence of I2 or CPM-I2.

The fluorescence method appears to be very well suited for the study of I2 interaction with PP1-C, reactivation by kinase F_A of the inactive I2-PP1-C complex, and inhibition of PP1-C by okadaic acid. The concordance of the K_i and K_d values

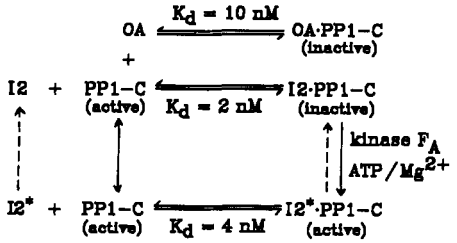

FIGURE 5: Scheme of the interrelationships between PP1-C, OA, I2, and kinase F_A. I2* designates the form of I2 which is represented in these studies by the high anisotropy form of CPM-I2. The broken arrows show the spontaneous reversal of the effect of kinase F_A and ATP/Mg^{2+}. This reaction is characterized by a decrease in CPM-I2 anisotropy and is slow relative to the kinase F_A mediated forward reaction.

indicates that the same phenomenon is observed by changes in fluorescence and by enzymatic activity. The results are summarized in Figure 5. They indicate that the free forms of I2 and PP1-C equilibrate with an enzymatically inactive I2·PP1-C complex, which for native I2 has a K_d of 2 nM. Kinase F_A plus ATP/Mg^{2+} activates the complex for phosphatase activity and causes a 2-fold increase in the K_d of the I2·PP1-C complex. This relatively small change in K_d is accompanied by an increase in the fluorescence quantum yield and anisotropy of the CPM-I2 whether bound or released. The most likely explanation for this increase is a conformational change within I2 itself which changes the local environment of the coumarin probe and restricts its movement relative to I2. This effect of the kinase F_A appears to occur only in the I2·PP1-C complex. However, by carrying out the reaction with kinase F_A under conditions of limiting PP1-C (Figure 3), free I2* can be generated in the reaction mixture by dissociation of the I2*·PP1-C complex. For CPM-I2, this form has high fluorescence anisotropy and quantum yield and can readily reassociate with PP1-C to yield an enzymatically active complex. The quantum yield and anisotropy of free I2* decline slowly under the conditions used ($t_{1/2} \cong 25$ min), apparently reflecting relaxation to the native I2 state or condition. The results from experiments in which native I2 or OA was used to displace the CPM-I2 from the complex with PP1-C led to the same conclusion.

Previously, a number of models have been proposed to describe how kinase F_A might activate the enzymatically inactive PP1-C·I2 complex (Villa-Moruzzi et al., 1984; Li et al., 1985; Vandenheede & Merlevede, 1985). Villa-Moruzzi and coworkers (Villa-Moruzzi et al., 1984) reported experiments in which inactive, free PP1-C (no I2), which had been isolated from the PP1-C·I2 complex, was activated directly by Mn^{2+}. The free inactive PP1-C could not be activated by kinase F_A and ATP/Mg^{2+} but was activated by the kinase when it was associated with I2. These observations led to the suggestion that phosphorylation of I2 caused enzymatic activation because of an induced conformational change in PP1-C, which decayed with time at a rate in the same order as we have observed for the relaxation of I2*. The results presented here do not preclude the possibility that activation is associated with an induced conformational change in both PP1-C and I2. However, the observation that kinase F_A action has no detectable effect on the K_d of the PP1-C·OA complex indicates that the F_A-induced change in PP1-C is not reflected in this parameter.

It should be noted that the data presented here do not provide a basis for unequivocally determining whether relaxation occurs in free I2*, the I2*·PP1-C complex, or both. Relaxation in the presence of a large excess of OA suggests that it occurs in free I2*; however, the rapid exchange between free and bound I2 dictates caution in reaching this conclusion.

The nature of the extremely interesting change in the state or conformation of I2 is unclear. I2 is heat-stable, resistant to many denaturants, and relatively protease-sensitive and has a Stokes radius of 3.5 nm (Foulkes & Cohen, 1980). Consideration of the Stokes radius of I2 with its molecular weight (22 922; Holmes et al., 1987) indicates that the I2 molecule has a high axial ratio in solution. These are the anticipated characteristics of a protein with little or no higher order structure. A kinase F_A dependent conversion to a more globular structure would be expected to lower the axial ratio of the whole molecule. This would be expected to decrease the rotational relaxation time of the protein which, in itself, would lead to a decrease rather than an increase in fluorescence anisotropy. It is for this reason that we suggest the increase in anisotropy may be related to a restriction in the movement of the probe itself, relative to the mass of the I2 protein. In any event, the results presented appear to provide an example of a conformational change in a protein that is coupled to a phosphorylation event.

Okadaic acid and CPM-I2 appear to act as mutual competitors for binding to PP1-C. However, treatment of the PP1-C·CPM-I2 complex with kinase F_A and ATP/Mg^{2+} does not change the K_d of the OA·PP1-C complex. This also is consistent with the conclusion that the direct effect of the kinase F_A is on the bound inhibitor rather than on PP1-C. The relationship of the I2 and the OA binding sites on PP1-C is unknown. OA is an even more potent inhibitor of type 2A phosphatases than of PP1-C, while I2 has little or no effect on the former.

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Differences in Deoxyribonuclease I Hypersensitive Sites in Phenobarbital-Inducible and Constitutive Rabbit P450IIC Genes[†,‡]

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ABSTRACT: DNase I hypersensivity of nuclear chromatin near the rabbit cytochrome P450IIC genes was investigated by indirect end-labeling genomic Southern analysis. Major DNase I hypersensitive sites were observed in proximal (−200 base pairs) and distal (−2000 to −2200 base pairs) regions of the genes. The presence of the proximal site correlated well with the expression states of the individual genes. In contrast, the distal site was present in DNA from both liver and kidney nuclei and in untreated and phenobarbital-treated animals irrespective of the expression state of the genes. However, the distal site was present only in genes that respond to phenobarbital (cytochromes P450IIC1 and P450IIC2) and was not detected in the constitutive cytochrome P450IIC3 gene. The nucleotide sequences of 500 base pairs in the distal site regions of cytochromes P450IIC1 and P450IIC2 were 67% similar, and hepatocyte nuclear factor 1 like motifs were conserved in these two sequences. These results are consistent with the hypothesis that distal regions cooperate with the proximal promoter regions in the regulation of cytochrome P450IIC gene expression.

Cytochrome P450 (P450)[1] genes encode microsomal hemoproteins that are terminal monooxygenases involved in the metabolism of many xenobiotic and endogenous substances (Nebert & Gonzalez, 1987). On the basis of nucleotide and amino acid similarity, the P450s form a multigene superfamily containing 27 families of which 10 are present in mammalian species (Nebert et al., 1991). This classification roughly correlates with the induction of P450 by various compounds.

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[‡] The nucleic acid sequences in this paper have been submitted to GenBank under Accession Numbers M76596 and M76597.
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[1] Abbreviations: P450, cytochrome P450; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,-*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; kbp (kb in figures), kilobase pair(s); bp, base pair(s).

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