Mass Spectrometric Determination of the Cleavage Sites in *Escherichia coli* Dihydroorotase Induced by a Cysteine-specific Reagent*

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Régis Daniel‡§, Eliane Caminade‡, Annie Martel‡, François Le Goffic‡, Daniel Canosa‡, Montse Carrascal¶, and Joaquim Abian¶

From the ¤Laboratoire de Bioorganique et Biotecnologies associé au Centre National de la Recherche Scientifique, Ecole Nationale Supérieure de Chimie de Paris, 11 rue P. & M. Curie, 75231 Paris cedex 05, France and ‡Consejo Superior de Investigaciones Científicas, Department of Medical Bioanalysis, Jordi Girona 18-26, 08034 Barcelona, Spain

*Escherichia coli* dihydroorotase contains six cysteines/subunit, which are potential ligands of structural and catalytic zinc metals at protein sites of the enzyme. Specific thiol reagents modify, in non-denaturing conditions only, two of these cysteines; these two residues are thought to be ligands of structural zinc. We report here on the localization of these two cysteines on the polypeptide chain through their cyanylation by 2-nitro-5-thiocyanobenzoic acid (NTCB) and the analysis by mass spectrometry of the protein adducts. This is the first study of *E. coli* dihydroorotase by mass spectrometry, allowing the accurate determination of the subunit molecular weight (38,695). Treatment of dihydroorotase by NTCB induced a cleavage N-terminal to the cyanlated cysteines. The resulting fragments visualized on electrophoresis gel have been N-terminal sequenced, and their masses were determined by electrospray-ionizing mass spectrometry. This allowed the identification of cysteines 221 and 265 as the two residues cyanylated by the reagent NTCB. Results from gel filtration of dihydroorotase cyanylated on the two cysteines indicate that these residues are involved in subunit interactions leading to the active dimer. Consistent with literature data, we assume that cysteine 221 and cysteine 265, along with the neighboring cysteines 263 and 268 arranged in cluster, are potent ligands of structural zinc of *E. coli* dihydroorotase.

The enzyme 1,5,6-dihydroorotase amidohydrolase (DHOase1; E.C. 3.5.2.3) catalyzes the key reaction of formation of t-dihydroorotate, the first cyclic intermediate in the de novo pathway of pyrimidine biosynthesis (1). Although DHOase is ubiquitous, distinct properties feature the prokaryotic enzymes from the eukaryotic ones, indicating an evolution of this class of enzymes in two different groups (2). Unlike most of the higher eukaryotic DHases, the bacterial and yeast enzymes are monofunctional proteins. The DHOase-encoding gene from *Escherichia coli* has been cloned and overexpressed (3).

The *E. coli* DHOase is a homodimer of 348 amino acids/subunit as deduced from the cDNA sequence (4, 5). Each subunit binds tightly one zinc atom, which has been suggested to be directly involved in catalysis, because its presence is required for the enzyme to be functional. In addition to this catalytic zinc, two other zinc atoms may bind weakly to each subunit. Lost during purification, they can be reincorporated by dialysis of the one zinc/subunit dihydroorotase against ZnCl2.

Substitution of Co(II) for the active site Zn(II) resulted in an active Co-DHOase. Its electronic absorption spectrum exhibited a broad charge transfer band that was attributed to a thiolate ligand (7). Among the six cysteines of each *E. coli* subunit, two are readily accessible to sulfhydryl reagents. The properties of the resulting cysteine-modified enzyme indicated that these two cysteines might be potential ligands to the structural Zn(II) in external protein sites.

We report here on the localization of these two reactive cysteines along the peptide chain of the DHOase subunit by selective chemical cleavage at the N-peptide bonds of cyanlated cysteine residues and electrospray-ionizing mass spectrometry analyses of the produced peptide fragments. 2-Nitro-5-thiocyanobenzoic acid (NTCB) is known to be an efficient reagent for selective cyanylation of cysteine residues in protein under mild conditions (8). NTCB is of great value for protein primary structure investigation compared with other cysteine reagents, because it has been shown that cyanylated polypeptides undergo an intramolecular cleavage at the amino peptide bond adjacent to the SCN-cysteiny residue upon incubation under slightly alkaline conditions (Scheme 1) (9).

MATERIALS AND METHODS

Materials—DHOase substrates N-carbamyl-L-aspartate and t-dihydroorotase, and the cysteine reagents DTNB and NTCB were purchased from Sigma. Other chemicals and reagents were obtained from commercial sources at the highest level of purity available. All buffers were prepared with ultrapure water (Biblock) and degassed before use.

Enzyme Assays—*E. coli* DHOase was purified to homogeneity as described previously from an overproducing strain (RLM 569) that overexpresses the *pyrC* gene cloned in plasmid pRK16 (6). The purified DHOase contained one equivalent of zinc/subunit. Activity was routinely assayed using the UV determination of DHO at 230 nm (ε<sub>230</sub> = 1170 M<sup>-1</sup> cm<sup>-1</sup>) (10) in the biosynthetic conversion of CA to DHO as well as in the degradative pathway of DHO into CA as reported previously (11). Protein concentrations were determined by the Bio-Rad dye binding method with bovine serum albumin as the standard (12) and referred to concentrations of subunits (i.e. active sites).

DTNB and NTCB Treatments of DHOase—The cysteine derivatization reaction was performed at 30 °C in 0.1 M Tris phosphate buffer, pH 8, containing 200 μM DTNB or NTCB and was initiated by the addition of DHOase (7.7–12.9 μM). For the determination of total cysteine,
Increasing the pH of the reaction mixture to 9.5 with 1 N NaOH followed the reaction with NTCB, the S₄₁₂ nm due to the release of the dianion TNB₂, monitored until completion by following the change in absorbance at DHOase was incubated with 6 M guanidine HCl. The reaction was determined from the final absorbance (equation A) and eluted with the mixture of water: acetonitrile: trifluoroacetic acid 95:5:0.05 (solvent A) and eluted with the mixture of water: acetonitrile: trifluoroacetic acid 10:90:0.045 (solvent B), using the following gradient program: 0% B, 2 min; 0–40% B, 4 min; 40–70% B, 10 min; 70–100% B, 1 min; 100% B, 1 min; 100% B, 1 min (flow rate of 0.3 ml min⁻¹). Purified fractions were evaporated to dryness in a Speed Vac concentrator and redissolved in water: methanol 1:1. A Finnigan (San Jose, CA; TSQ-700 triple stage quadrupole mass spectrometer provided with a Finnigan ESI/APCI ion source was used for molecular weight determination. The proteins were ionized by the electrospray method, and the instrument electrical potentials were set for positive ion detection. Mass spectrometry conditions were as follows: electrospray needle voltage, 4.5 kV; sheath gas pressure (nitrogen), 30 psi; electron multiplier voltage, 1200; dinode voltage, 15 kV; ion acquisition range, m/z 400 to m/z 2000. After acquisition, the molecular weight of the protein was obtained from the electrospray spectrum by using the deconvolution software provided by Finnigan. The molecular weights given in the text are the average values resulting from at least three analyses of each sample. Each spectrum shown is one of those corresponding to these analyses.

**RESULTS**

**Molecular Weight of the DHOase Subunit**—The preparation of the purified DHOase used for mass spectrometric studies was analyzed by fast protein liquid chromatography gel filtration and SDS gel electrophoresis. The DHOase, eluted in one peak from the filtration column, had an apparent molecular weight of 77,700. The active fractions SDS-PAGE migrated on a 12.5% polyacrylamide gel as a single band of apparent molecular weight 38,600. These values were in agreement with previous work on native and cloned DHOase (6, 16), which showed the homodimeric structure of the E. coli DHOase. After SDS-PAGE, the band corresponding to the DHOase subunit was transferred to a nylon membrane, and its N-terminal sequence was determined. The first 12 amino acids were those deduced from the cDNA sequence (5), except that the N-terminal methionine was not detected as reported for the native DHOase (16). The molecular weight of the DHOase subunit was thus calculated from the amino acid composition deduced from the cDNA sequence starting from the N-terminal threonine (second residue of the cDNA sequence). The resulting calculated molecular weight of the 347-amino acid DHOase subunit was 38,695. Mass spectrometric analysis of the purified DHOase gave a molecular weight of 38,695 ± 5 (n = 6) (Fig. 1), in excellent agreement with the theoretical value. A very minor component of 38,728 was sometimes observed, which may correspond to the enzyme with a blocked N terminus, as reported previously by Brown and Collins (7).

**DTNB and NTCB Treatment of DHOase**—During incubation of active DHOase with DTNB, 2 mol of diaminon TNB²⁻/subunit were released, indicating the oxidative modification of two cysteines among the six of the enzyme. All six cysteines were modified by NTCB when the incubation was carried out in 6 M guanidine HCl. Cyanlated cysteines rather than mixed disulfide are formed upon incubation with NTCB, but the extent of modification can be determined in the same way (i.e. through the quantitation of the released diaminon TNB²⁻). As in the case of DTNB, two cysteines of the active DHOase were modified by NTCB (Fig. 2). No cysteine is labeled by NTCB when the reaction is carried out on DHOase modified previously by DTNB and vice versa. Consequently, both reagents DTNB and NTCB react with the same two cysteines of the DHOase subunit. The mass spectrometric analyses of the NTCB-treated DHOase confirmed that two cysteines were cyanlated (Fig. 3). Indeed, an average molecular weight of 38,753 ± 5 (n = 6) was obtained for the cyanlated DHOase, in

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**Scheme 1. Cyanlation reaction of cysteine residue in a polypeptide chain and cleavage N-terminal to the cyanlated cysteine in slightly alkaline conditions.** The resulting N terminus is blocked by the iminothiazolidine carboxylyl group (in bold), which impedes Edman degradation sequence analysis.

**Analytical Procedures**—Gel filtration was performed by fast protein liquid chromatography on a Superose 12 column (Pharmacia Biotech, Inc.) eluted with 0.1 M phosphate buffer, pH 7.5, and calibrated with a kit of marker proteins (Pharmacia; 12–700 kDa). Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed on 12.5% polyacrylamide gel according to the method of Laemmli (14) using a vertical slab electrophoresis unit (model Mini-Protean II, Bio-Rad). Proteins were blotted by electrotransfer (1 h, 100 V) from the gel to an Immobilon P membrane (Whatman) as described by Towbin et al. (15) using a Mini Trans-Blot cell (Bio-Rad). After transfer, the membranes were rinsed with ultrapure water, rapidly saturated with 100% methanol, and stained for 1 min with a solution of 0.1% Amido Black, then washed several times in ultrapure water, and after air drying, the blue bands were excised for subsequent N-terminal sequence determination. The blotted proteins were subjected to automated Edman degradation with an Applied Biosystems Procise 492 protein sequencer.

**Mass Spectrometric Analyses**—The protein samples were made free of salts and buffers by an high performance liquid chromatography purification step on an Hypersil C4 column before mass spectrometric analyses. This high performance liquid chromatography procedure was also used to isolate the two fragments 29,000 and 25,000 from the rest of the cleavage mixture. An Applied Biosystems chromatographic apparatus equipped with a syringe pump and an UV detector was used. UV detection of the eluted material was done at 214 nm. The column was equilibrated with a mixture of water: acetonitrile: trifluoroacetic acid 95:5:0.05 (solvent A) and eluted with the mixture of water: acetonitrile: trifluoroacetic acid 10:90:0.045 (solvent B), using the following gradient program: 0% B, 2 min; 0–40% B, 4 min; 40–70% B, 10 min; 70–100% B, 1 min; 100% B, 1 min; 100% B, 1 min (flow rate of 0.3 ml min⁻¹). Purified fractions were evaporated to dryness in a Speed Vac concentrator and redissolved in water: methanol 1:1. A Finnigan (San Jose, CA; TSQ-700 triple stage quadrupole mass spectrometer provided with a Finnigan ESI/APCI ion source was used for molecular weight determination. The proteins were ionized by the electrospray method, and the instrument electrical potentials were set for positive ion detection. Mass spectrometry conditions were as follows: electrospray needle voltage, 4.5 kV; sheath gas pressure (nitrogen), 30 psi; electron multiplier voltage, 1200; dinode voltage, 15 kV; ion acquisition range, m/z 400 to m/z 2000. After acquisition, the molecular weight of the protein was obtained from the electrospray spectrum by using the deconvolution software provided by Finnigan. The molecular weights given in the text are the average values resulting from at least three analyses of each sample. Each spectrum shown is one of those corresponding to these analyses.
close agreement with the theoretical value of 38,746 expected for the enzyme with two covalently attached CN groups.

Cleavage of DHOase at S-Cyanylated Cysteine Residues—The cleavage reaction was performed by adjusting the cyanylation reaction mixture to pH 9 and then incubating it at 37 °C for 17 h. SDS-PAGE analysis showed, besides the cyanylated DHOase, two new bands of lower molecular weight that increased in intensity during the first 10 hours of reaction (Fig. 4). Incubation at 37 °C for periods longer than 17 h did not increase the intensity of these bands. After 17 h of incubation, DHOase still was the major band, indicating a limited cleavage reaction as reported previously for other proteins (17). The two new bands migrated at apparent molecular weights of 29,000 and 25,000. The N-terminal sequences of these two components as well as of the cyanylated DHOase were determined after electrophoretic transfer of the protein bands. All of them had the same N-terminal sequence as the one obtained above for the native DHOase. This indicated that the two peptides of Mr 29,000 (peptide A) and Mr 25,000 (peptide B) stemmed from the DHOase subunit and that they resulted from the removal of C-terminal fragments of approximately 8,000 and 13,000, respectively (according to the mechanism of the cleavage reaction shown on Scheme 1, these two small fragments should have blocked N termini; this is confirmed by unsuccessful attempts to sequence the N terminus of the fragment 13,000. Purification and characterization of these two peptides are currently in progress). Given the location of the six cysteines on the DHOase subunit polypeptide chain, few cleavage sites are possible. Concerning the peptide A, only a cleavage at one of the three cysteines arranged in a cluster at positions 263, 265, and 268 (Scheme 2) could result in a peptide of this mass. Regarding peptide B, only a cleavage at cysteine 221 can give a fragment of this molecular weight. Consequently, cysteine 221 and one of the three cysteines 263, 265, or 268 should be the site of cyanylation by NTCB.

Mass Spectrometric Analysis of the Cleavage Reaction—The mass spectrum of the mixture of the two peptides A and B, separated previously from the uncleaved DHOase by reversed-phase high performance liquid chromatography, exhibited as expected two components (Fig. 5). The first one had a
molecular weight of 29,631 $\pm 5$ ($n = 9$) and thus should correspond to peptide A. A molecular weight of 24,684 $\pm 3$ ($n = 9$) was determined for the second peak, which was then attributed to peptide B. We assumed above that the latter resulted from the cleavage of DHOase at cysteine 221, generating a peptide of calculated $M_r$ 24,684. The molecular weight obtained by mass spectrometry for peptide B is thus in agreement with the calculated value. The results from mass spectrometry and N-terminal sequencing of peptide B indicate cysteine 221 as one of the two cysteines cyanylated by NTCB.

We assumed above that a cleavage at one of the three cysteines 263, 265, or 268 generated peptide A, which contains cysteine 221. Because this cysteine 221 is cyanylated, the mass of one CN group has to be taken into account in the calculated molecular weight of the peptide A. The N-terminal peptide produced by a cleavage N-terminal to cysteine 265 and cyanylated at cysteine 221 has a calculated molecular weight of 29,605, which is very close to the value of 29,631 obtained by mass spectrometry. Actually, the difference (+26) between the two values fits exactly with the mass of one CN group, indicating that an extra cyanylation of the peptide occurs after the cleavage at cysteine 265. Cysteines 78 and 263 are the remaining potential sites for this additional cyanylation. A signal at a molecular weight of 29,488 could be seen in the mass spectrum (Fig. 5) close to the peak corresponding to peptide A. A cleavage of this peptide A at the position N-terminal to cysteine 263 yields a peptide of this range of molecular weight (Scheme 2), suggesting that this residue 263 could be the site of the additional cyanylation. It is likely that cysteine 263 becomes more

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**Fig. 3.** ESI mass spectrum of NTCB-treated E. coli dihydroorotase. This NTCB-treated dihydroorotase contained two CN groups (determined as in Fig. 2), in agreement with the mass increase of its peak represented in the deconvoluted spectrum.

**Fig. 4.** Cleavage of E. coli dihydroorotase subunit analyzed by SDS-12.5% polyacrylamide gel electrophoresis. Lane A, molecular weight markers. Lane B, native E. coli dihydroorotase (30 µg). Lanes C and D, NTCB-treated E. coli dihydroorotase 0.5 and 17 h, respectively, after the beginning of the cleavage reaction. After treatments with NTCB, the dihydroorotase contained two cyanylated cysteines/subunit. Cleavage reaction was initiated at the end of the incubation with NTCB by increasing pH of the incubation mixture as described under "Materials and Methods."
exposed to NTCB after cleavage N-terminal to the neighboring cysteine 265. Taking into account this additional cyanlation, the data from gel electrophoresis, N-terminal sequencing, and mass spectrometry indicate that, besides cysteine 221, cysteine 265 is the other primary site of cyanlation of \textit{E. coli} DHOase under nondenaturing conditions.

\section*{DISCUSSION}

This paper reports the first study of \textit{E. coli} dihydroorotase by mass spectrometry. This powerful analytical method allowed an accurate determination of the molecular weight of the DHOase subunit, compared with the previous estimations by SDS-PAGE and sedimentation equilibrium analyses (16). The DHOase subunit has a molecular weight of 38,696, \textit{i.e.} the mass of the 347-amino acid peptide chain beginning from threonine 2 to C-terminal glutamine 348 on the sequence deduced from cDNA. This is in agreement with the N-terminal sequencing, which identifies threonine 2 as the N-terminal residue. Quantitative analyses indicated that the amount of sequenced N-terminal extremities was as high as expected from the concentration of the applied sample. Therefore, our DHOase preparation exhibited no blocked N terminus, indicating a complete removal of the N-(formyl)methionine.
The mass spectrometry study of the modified DHOase confirms also that two cysteines among the six of the DHOase subunit are easily accessible to specific thiol reagents under non-denaturing conditions, suggesting exposed, external positions for these two residues (6). The features of the cleavage reaction induced by NTCB combined with mass spectrometric analysis provide an efficient method to locate cysteines on a polypeptide chain, as reported recently (18). Applying this strategy to DHOase, we identified these two exposed thiol residues as cysteines 221 and 265. Cysteine is the most common protein ligand for structural zinc (19). Given the difference of sensitivity between the one zinc/subunit and the three zincs/subunit DHOases toward air oxidation and thiol reagents (6, 7), we assume that the two cysteines 221 and 265 targeted by DTNB and NTCB are ligands of the external (structural) zinc ion. As observed previously by Brown and Collins (7), cysteine 265 belongs to a cluster of three cysteines (263, 265, and 268), which have the same spacing as three of the four cysteine ligands of one of the domains of metallothionein (20). Accordingly, cysteines 263 and 268 could be also considered as potential ligands of structural zinc. These two residues are not accessible to thiol reagents in native DHOase likely for steric hindrance because at least one of these is cyanurated upon cleavage reaction.

Structural zinc atoms in proteins are often in outlying regions of the macromolecules, where they affect the local structure and conformation (21). Structural zincs are also involved in stabilization and protection of the protein against oxidation, likely by a very limited access to their protein binding domains (22). Washabaugh and Collins (6) showed that the three zincs/subunit DHOase was protected against air oxidation as compared with the one zinc/subunit and that air oxidation was reversed by addition of dithioerythritol. Air-oxidized DHOase has a decreased specific activity and does not react with DTNB and NTCB. As a consequence, the two cysteines 221 and 265, which are targeted by the thiol reagents, are also the sites of the oxidative modification. These authors suggested that cysteines of air-oxidized DHOase were oxidized to either a sulfenic acid or a disulfide. We did mass spectrometry analysis of air-oxidized DHOase and obtained a molecular weight of 38,694, almost identical to the molecular weight of the native DHOase (data not shown). This result favors the hypothesis of formation of a disulfide bond.

To check the effect of the modification of the two cysteines 221 and 265 on the overall structure of the DHOase subunit, we are performing studies by gel filtration and circular dichroism that will be published later. It is worthwhile to note our preliminary results, which show that DTNB- or NTCB-modified DHOase is eluted on gel filtration columns under non-denaturing conditions as a dimer and partially as a monomer. No monomer is observed for the native DHOase, which is eluted as a single peak of dimer (Fig. 6). Therefore, modification of the two cysteines disrupts the conformation of the DHOase subunit in such a way that formation of the dimer is partially impeded. The partial formation of the monomer parallels the limited cleavage induced by NTCB. It seems from literature data that the proteins retain the SCN groups without cleavage, so long as the native structure is retained (9). The SCN group must be oriented appropriately for nucleophilic attack, and such an orientation may be constrained by the native protein structure. Then the hypothesis could be raised that the monomer fraction is the same fraction cleaved upon incubation at alkaline pH.

Finally, cysteines 221 and 265 are the two thiol residues modified by DTNB and NTCB, and it is likely that these two cysteines as well as potentially cysteines 263 and 268 are ligands of the structural zinc atoms. The latter stabilize DHOase through protection of these thiol residues against air oxidation and contribution to protein conformation to yield the active dimer. Concerning the catalytic zinc, previous studies on Co(II)-substituted E. coli DHOase implicated a thiol group as a ligand at the active site (7). The two remaining cysteines not targeted in this study were cysteines 78 and 295. The former is located on the primary sequence between the most conserved protein segment, which contains the histidine residues 16 and 18 and the region containing histidine 139. These three conserved histidines have been recently shown to be ligands of the catalytic zinc at the active site of mammalian DHOase by site-directed mutagenesis (23–25). We are currently investigating the possible role of cysteines 78 and 295 as zinc ligands at the active site of E. coli DHOase.

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REFERENCES
1. Carrey, E. A. (1995) Biochem. Soc. Trans. 23, 899–902
2. Simmer, J. P., Kelly, R. E., Rinker, A. G., Jr., Zimmermann, B. H., Scully, J. L., Kim, H., and Evans, D. R. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 174–178
3. Brown, D. C., and Collins, K. D. (1986) J. Biol. Chem. 261, 5917–5919
4. Backes, M. S., and Dong, L. (1986) Eur. J. Biochem. 160, 77–82
5. Wilson, H. R., Chan, P. T., and Turnbaugh, C. L., Jr. (1987) J. Bacteriol. 169, 3053–3058
6. Washabaugh, M. W., and Collins, K. D. (1986) J. Biol. Chem. 261, 5920–5929
7. Brown, D. C., and Collins, K. D. (1991) J. Biol. Chem. 266, 1597–1604
8. Degani, Y., and Patchornik, A. (1974) Biochemistry 13, 1–11
9. Stark, G. R. (1977) Methods Enzymol. 47, 129–132
10. Sander, E. G., Wright, L. D., and McCormick, D. B. (1965) J. Biol. Chem. 240, 3626–3630
11. Daniel, R., Kelkel, B., Caminade, I., Martel, A., and Le Goffic, F. (1996) Anal. Biochem. 239, 130–135
12. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
13. Riddle, P. W., Blakeley, R. L., and Zerner, B. (1979) Anal. Biochem. 94, 75–81
14. Laemmli, U. K. (1970) Nature 227, 680–685
15. Towbin, H., Staehlin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
16. Washabaugh, M. W., and Collins, K. D. (1984) J. Biol. Chem. 259, 3293–3298
17. Ogilvie, J. W. (1980) Biochim. Biophys. Acta 610, 127–128
18. Wu, J., Gage, D. A., and Watson, J. T. (1996) Anal. Biochem. 235, 161–174
19. Christianson, D. W. (1991) Adv. Prot. Chem. 42, 281–355
20. Christianson, D. W., and Alexander, R. S. (1989) J. Am. Chem. Soc. 111, 2506–2512
21. Vallee, B. L., and Auld, D. S. (1990) Biochemistry 29, 5647–5659
22. Gibbons, P. N., and Jordan, P. C. (1981) Biochem. Soc. Trans. 9, 232–233
23. Williams, N. K., Manthey, M. K., Hambley, T. W., O'Donoghue, S. I., Keegan, M., Chapman, B. E., and Christopherson, R. I. (1995) Biochemistry 34, 11344–11352
24. Zimmermann, B. H., Kemling, N. M., and Evans, D. R. (1995) Adv. Exp. Med. Biol. 370, 699–702
25. Zimmermann, B. H., Kemling, N. M., and Evans, D. R. (1995) Biochemistry 34, 7038–7046