Excess Nucleoside Triphosphates (or Zinc) Allow Recovery of Alkaline Phosphatase Activity Following Refolding under Reducing Conditions †

ARTICLE *in* BIOCHEMISTRY · DECEMBER 1998

Impact Factor: 3.02 · DOI: 10.1021/bi972833g · Source: PubMed

3 AUTHORS, INCLUDING:

Sailendra Nath Sarkar
University of Calcutta
18 PUBLICATIONS **221** CITATIONS

Excess Nucleoside Triphosphates (or Zinc) Allow Recovery of Alkaline Phosphatase Activity Following Refolding under Reducing Conditions†

Nandini Ghosh,‡ Sailendra Nath Sarkar,‡ and Kunal B. Roy*,§

Department of Biochemistry and Biophysics, Kalyani University, Kalyani 741235, and Centre for Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

Received November 19, 1997; Revised Manuscript Received August 13, 1998

ABSTRACT: The contribution of ATP and other nucleotides to the stabilization of non-native structures has been described for some proteins. We report here the effect of GTP, ATP, and their nonhydrolyzable analogues on the denaturation and renaturation of the enzyme *Escherichia coli* alkaline phosphatase. We show that GTP, ATP, and their nonhydrolyzable analogues considerably stimulate renaturation of AP in the presence of 2-mercaptoethanol where spontaneous renaturation is completely arrested due to reduction of S−S bonds. GTP is the most efficient inducer of reconstitution of the active site and appears to play a specific role besides being a substrate. The reconstituted protein was found to be in the reduced form despite having near-normal activity. The self-refolded oxidized form and the GTP-refolded reduced form had the same K_M/k_{cat} values and showed similar structural properties. We conclude that GTP can not only induce reconstitution of dimerization-competent monomers because of its substrate nature but also act as a modulator of the activity of AP. We also report here on the Zn^{2+}-assisted reconstitution of *E. coli* AP under reducing condition. The prior formation of a disulfide bond for positioning the active site residues in the proper geometry is unnecessary under this condition.

The contribution of ATP to the stabilization of the native structure was described for some proteins. Sadis et al. (*1*) showed that ATP, ADP, GTP (but not AMP), and also nonhydrolyzable analogues AMPPCP[1] and ATP[γS] prevented heat-induced aggregation of hsp73 and dnaK protein. The two proteins have specific ATP binding sites, and the authors concluded that the mere binding of purine nucleoside triphosphate or diphosphate was sufficient to stabilize these proteins against heat-induced aggregation. Goto et al. (*2*), on the other hand, reported that ATP and other nucleotides have strong potentials to stabilize the molten globule state of two proteins at pH 2.0 for which they are not specific ligands. They also demonstrated stabilization of structure by ATP for a synthetic peptide at neutral pH, which forms a helix−turn−helix motif. The authors, however, suggested the effective role of ATP as a multivalent anion. In this work, we report the effect of ATP and GTP on the denaturation and renaturation of the enzyme alkaline phosphatase (AP) from *Escherichia coli* for which the nucleotides are only weak substrates as found from the measurements of the rates of inorganic phosphate release.

Alkaline phosphatase of *E. coli* exists in the periplasmic space as a dimer of identical subunits. Each monomer contains an active site that includes the hydroxyl group of Ser102 involved in catalysis and three cation binding sites which bind two Zn^{2+} ions and one Mg^{2+} ion (*3*). The active AP contains two intramolecular S−S bonds in each monomer (*4*). The formation of S−S bonds is thought to be a prerequisite for the proper folding and activation of AP in vivo (*5*−*7*) as well as in vitro (*8*, *9*). Sone et al. (*10*) recently showed that one S−S bond is required and is sufficient for correctly positioning the active site region of this enzyme; however, such an active conformation is still insufficient for the conformational stability of the enzyme, and the formation of two S−S bonds is necessary for the formation of a fully active, stable enzyme. In a previous paper, we reported that renaturation of acid-denatured or GdnCl-denatured AP by self-folding could be completely arrested by including a thiol-reducing agent in the reactivation buffer and indicated that reactivation can be induced by Zn^{2+} or GTP even under reducing conditions (*11*). We have now systematically investigated the reactivation process and report that GTP and ATP as well as their nonhydrolyzable analogues allow recovery of AP activity following refolding under reducing conditions. We show here that GTP particularly is the strongest inducer of reconstitution of the active site. We also report that instead of NTPs inclusion of Zn^{2+} (15−20 μM) in the same reducing buffer restores the enzymatic activity to 40% of the native activity. We propose that GTP

† This work has been carried out with financial assistance from the Department of Science and Technology (Grant SP/SO/D11/92), Government of India. S.N.S. gratefully acknowledges DST for a Senior Research Fellowship, and N.G. gratefully acknowledges Jawaharlal Nehru University for a visiting professorship.

* To whom correspondence should be addressed: Centre for Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India. E-mail: kbroy@jnuniv.ernet.in. Fax: +91(011)6165886.

‡ Kalyani University.

§ Jawaharlal Nehru University.

[1] Abbreviations: AP, alkaline phosphatase; 1,8-ANS, 1-anilinonaphthalene-8-sulfonate; AMPPCP, adenyl (β-*y*-methylene)diphosphate; GMPPNP, guanyl (β-*y*-methylene)diphosphate; Gl-6-P, glucose 6-phosphate; GdnHCl, guanidine hydrochloride; 2-MCE, 2-mercaptoethanol; pNPP, *p*-nitrophenyl phosphate; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

10.1021/bi972833g CCC: $15.00
Published on Web 10/14/1998

first stimulates proper folding of dimerization-competent monomers by interacting with some structural elements of the substrate binding site. GTP then acts as a modulator of the dimer assembly by interacting at the dimerization interface.

MATERIALS AND METHODS

E. coli alkaline phosphatase (EC 3.1.3.1) as a lyophilized powder, guanidine hydrochloride (GdnHCl), 1-anilinonaphthalene-8-sulfonate (1,8-ANS), 2-mercaptoethanol (2-MCE), heparin, spermine, and spermidine were from Sigma Chemical Co. The lithium salts of ATP, GTP, AMPPCP, and GMPPNP were from Boehringer Mannheim. The nucleotide solutions were prepared fresh before being used. *p*-Nitrophenyl phosphate (pNPP) was from E. Merck. SDS−PAGE materials were from Bio-Rad. All other chemicals were collected locally and were of analytical grade.

Denaturation and Renaturation. Denaturation and renaturation of AP were carried out as described previously (*11*). Briefly, AP (0.5 mg/mL, 5.3 μM) was denatured in 6 M GdnHCl at 37 °C for 4 h, a time long enough to achieve a complete change in the parameters being followed. The denatured sample was diluted 40-fold in the reactivation buffer and the mixture incubated at 37 °C and assayed for enzymatic activity thereafter. The extent of denaturation and renaturation was expressed as a percentage of the native activity. The carryover GdnHCl in the reactivation buffer had no effect on the activity of the native enzyme.

Reactivation was carried out in the presence of NTPs or Zn^{2+} as the inducer as required. Reactivation buffer was 20 mM Tris-HCl (pH 7.5) and 10 mM magnesium acetate without or with 6 mM 2-MCE. The presence of nucleotides did not inhibit the rate of PNP production by the native enzyme competitively (an up to 3-fold excess tested).

K_M (micromolar) and V_{max} (micromoles per minute per milligram of protein) for native and the reconstituted proteins were calculated from Linewaever−Burk plots. The pNPP concentration range was from 20 to 400 μM.

Spectral Studies. All spectral measurements were taken at a protein concentration of 50 μg/mL. Fluorescence spectra were recorded with a Perkin-Elmer luminescence spectrometer (model LS-50 B), at room temperature (25 °C). The excitation wavelength for tryptophan fluorescence was 295 nm and for 1,8-ANS fluorescence 395 nm. CD measurements were taken with a Jasco-720 spectropolarimeter, at room temperature (25 °C), in a 0.1 cm path length cell, from 200 to 300 nm. Mean residue ellipticities (θ) were calculated on the basis of a mean residue molecular weight of 114. Details of the methodologies are given in ref *11*.

Analysis of the Redox State of AP. The redox state of AP was analyzed electrophoretically according to the method of Derman and Beckwith (*6*) with some modification. In these experiments, denaturation was carried out at a concentration of 15 mg/mL overnight and reconstitution was carried out at 375 μg/mL. The proteins reconstituted under various conditions (with and without 6 mM 2-MCE) were diluted 4-fold in the deaerated carboxymethylation buffer [1.5% SDS, 5 mM EDTA, and 100 mM Tris-HCl (pH 9.0) final concentrations] and boiled for 10 min. The mixture was treated with a freshly prepared solution of iodoacetamide (50 mM final concentration) and kept for 90 min under a nitrogen atmosphere at room temperature. The concentration of iodoacetamide in the SDS gel sample buffer was 42.5 mM [2% SDS, 15% glycerol/bromophenol blue, and 125 mM Tris-HCl (pH 6.8)]. For the preparation of oxidized and reduced markers, native AP was boiled in gel sample buffer without or with 100 mM 2-MCE, respectively. Samples were analyzed with 10% SDS−polyacrylamide gels.

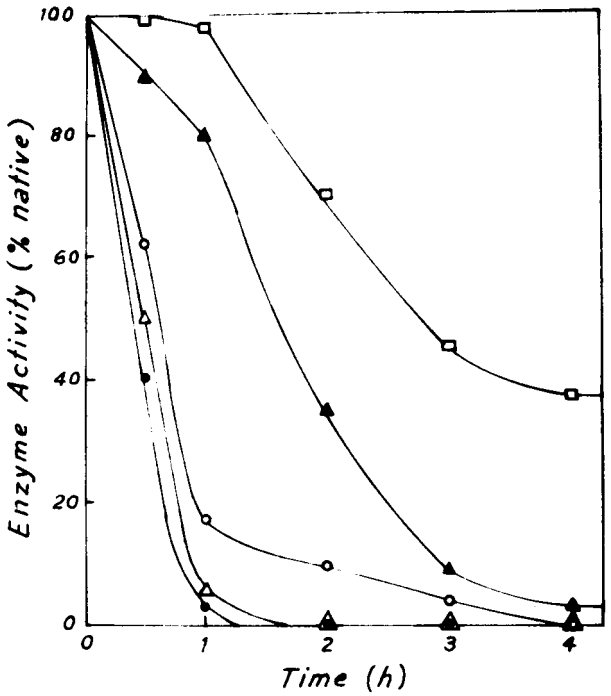

FIGURE 1: Kinetics of inactivation of AP in 6 M GdnHCl in the absence (●) and presence of Gl-6-P (□), GTP (▲), ATP (○), and GMPPNP and AMPPCP (△). The latter two curves were completely overlapping. The data presented are averages of three independent experiments.

Oxidation of Reduced Reconstituted AP. GTP-induced reconstituted AP (protein concentration of 12.5 μg/mL) in the reactivation buffer (with 4 mM GTP and 6 mM 2-MCE) was treated with 50 mM GSSG. One hundred microliters of this mixture was dialyzed against the buffer [20 mM Tris-HCl (pH 7.6), 10 mM magnesium acetate, 50 mM GSSG, and 4 mM GTP] with two changes, 1 mL each. The enzymatic activities before and after dialysis were determined. A similar experiment was carried out with 15 μM Zn^{2+}-induced reconstituted AP, and the results are given in Table 3.

RESULTS

Effect of NTPs on Denaturation. AP was denatured by treatment with 6 M GdnHCl. Figure 1 shows the rate of inactivation under a given experimental condition in the absence and presence of Gl-6-P and the NTPs. The rates of release of inorganic phosphate from Gl-6-P, GTP, and ATP by AP were found to be in the ratio 3.8:1.3:1.0 (S. N. Sarkar and N. Ghosh, unpublished data). Therefore, it appears that Gl-6-P, GTP, and ATP protected denaturation of AP in proportion to their efficiencies as substrates. GMPPNP and AMPPCP could not protect denaturation even at 16 mM.

NTP-Assisted Renaturation. AP was denatured in 6 M GdnHCl for 4 h and then diluted into the reactivation buffer [20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, and

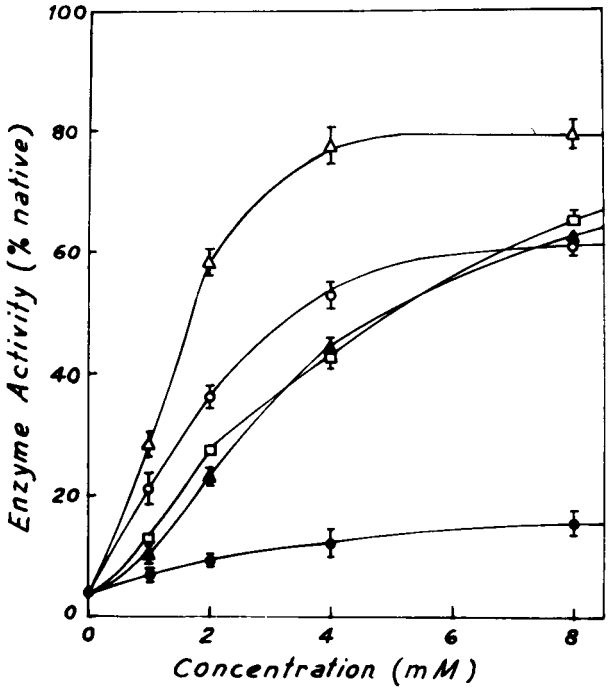

FIGURE 2: Effects of various substrates in the reactivation of 6 M GdnHCl-denatured AP. Substrates at the indicated concentrations were present in the reactivation buffer. Results are the average of six independent experiments: Gl-6-P (●), GTP (△), ATP (○), GMPPNP (□), and AMPPCP (▲).

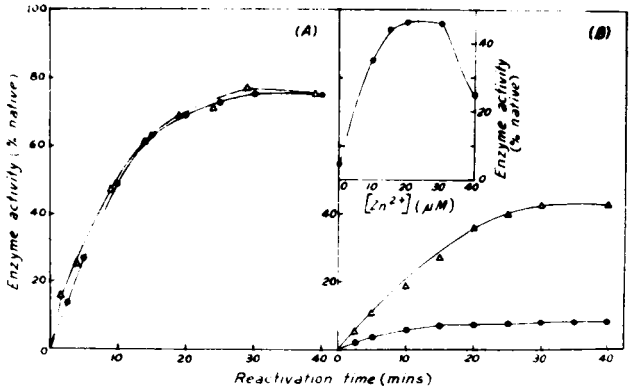

FIGURE 3: Time course of reactivation of 6 M GdnHCl-denatured AP in a nonreducing reactivation buffer (no MCE) (A) and in a reducing reactivation buffer (with 6 mM 2 MCE) (B) in the absence (●) and presence (△) of 15 μM $ZnCl_2$. The inset in panel B shows the effect of Zn^{2+} concentration on the reactivation, when reactions are carried out for 30 min.

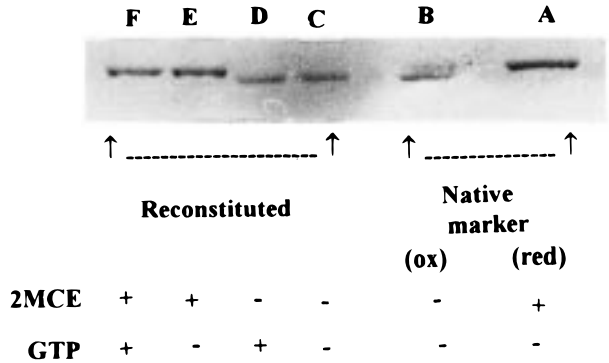

FIGURE 4: Analysis of the redox state of the native and reconstituted AP. See Materials and Methods for the details of the experimental procedure and Table 1 for reactivation conditions.

6 mM 2-MCE] containing Gl-6-P or various amounts of NTPs (Figure 2). Arrest of self-reactivation in the presence of 6 mM 2-MCE is obvious from the residual activity at a NTP concentration of zero. All the NTPs except Gl-6-P stimulated renaturation considerably, GTP being the most effective. The level of activity rescued at a particular concentration of GTP (or Zn^{2+}) could be further recovered by delayed addition of more GTP (but not Zn^{2+}). For example, the activity obtained at 2 mM GTP was 60% which could be raised to about 80% when additional GTP was added to make it 4 mM after 40 min (unpublished data, not shown). GTP also stimulated renaturation of AP denatured at pH 2.0, but only when spontaneous renaturation was totally inhibited by 2-MCE (*11*). We observed that nonsubstrate multivalent anion heparin or other multivalent cations spermine or spermidine did not stimulate renaturation of either GdnCl-denatured or pH-denatured AP in the presence of 2-MCE. The failure of heparin, spermine, and spermidine to mimick NTP indicates that the effect of the NTPs is specific, and not merely the result of generic electrostatic effects.

Spontaneous and Zn^{2+}-Assisted Renaturation. Denaturation was not affected by the presence of up to 40 μM Zn^{2+} during denaturation as seen from activity assays and fluorescence studies (data not shown). Figure 3A shows the time course of renaturation of the denatured AP in nonreducing reactivation buffer [20 mM Tris-HCl (pH 7.5) and 10 mM magnesium acetate] in the absence and presence of 15 μM Zn^{2+}. It is clear that the population with an intact S−S bond is capable of reconstituting the functional active site by self-refolding and the presence of extraneous Zn^{2+} has no stimulatory effect on this reconstitution. Figure 3B shows the kinetics of renaturation of a similarly denatured population in the reducing reactivation buffer [20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, and 6 mM 2-MCE]. It is obvious that in the reducing atmosphere the presence of extraneous Zn^{2+} dramatically induced the reconstitution of the active site, whereas in the absence of Zn^{2+}, this population failed to reconstitute the active site by self-folding. Zn^{2+}-assisted renauration led to only 40% of the native activity, which did not increase further at higher Zn^{2+} concentrations. However, delayed addition of GTP (2 mM final concentration) after renaturation for 40 min in the presence of 15 μM Zn^{2+} increased the level of activity to 60% after incubation for a further 30 min (unpublished data).

Analysis of the Redox State. The redox states of the native and reconstituted proteins were examined under similar conditions. Results with GTP are shown in Figure 4 and Table 1. It is evident that the protein self-refolded in the absence of 2-MCE was in the oxidized form (lane C) and inclusion of GTP during the self-refolding had no effect either on the redox state (lane D) or on the enzymatic activity of the refolded protein. When the reconstitution was carried out in the presence of 6 mM 2-MCE, the self-refolded protein was in the reduced form (lane E) with a concomitant loss in enzyme activity. However, when GTP was included in the reactivation buffer containing 6 mM 2-MCE, the refolded protein was able to reconstitute the enzymatic activity despite being in the reduced form (lane F). Similar experiments were carried out with the Zn^{2+}-reconstituted protein, and the results were also similar (data not shown). It was observed that

Table 1: Redox States of Native, Self-Refolded, and GTP-Refolded AP

AP	[2-MCE] (mM)	[GTP] (mM)	redox state	enzyme activity (% native)	lane no. of Figure 3
native	0	0	oxidized	100	B
native	100	0	reduced	0	A
native	0	4	oxidized	100	data not shown
self-refolded	0	0	oxidized	80	C
self-refolded	0	4	oxidized	80	D
self-refolded	6	0	reduced	5	E
GTP-refolded	6	4	reduced	80	F

Zn-assisted reconstitution resulted in a protein population which was still in the reduced state but exhibited 40% of the native activity. Sone et al. (*10*) demonstrated that the electrophoretic mobility of partially oxidized but active AP is indistinguishable from that of the oxidized wild type enzyme. GTP- or Zn-refolded active AP is not partially oxidized since its electrophoretic mobility is found to coincide with that of the reduced marker. It may be noted that we have also carried out experiments where IA was included (50 mM final concentration) in the carboxymethylation buffer itself to neutralize the carryover 2-MCE (1.5 mM) and prevent reduction of any residual S−S bonds. In these experiments, we also did not observe any oxidized form in the gel for the Zn^{2+}- or GTP-refolded protein (data not shown). We also observed that native AP at the same concentration in the reconstitution buffer containing 6 mM 2-MCE when treated exactly similarly, i.e., diluted 4-fold in the carboxymethylation buffer and boiled for 10 min, remained in the oxidized form.

Properties of Reconstituted AP. Some structural and kinetic properties of self-refolded (in the absence of 2-MCE) and GTP-refolded (in the presence of GTP and 2-MCE) AP are presented in Table 2. Analysis of the structural data shows that though the self-refolded oxidized form and the GTP-refolded reduced form resemble each other closely, they do differ somewhat from the native form. The native and the refolded proteins exhibited a fluorescence emission peak at 330 nm, indicating that the Trp residues are buried well within the protein structure. Upon complete denaturation, the peak was red shifted to 350 nm due to the transfer of Trp residues to a more polar environment (*11*). In Table 2, the ratios of emission intensities at these wavelengths (I_{330}/I_{350}) are presented so the overall global conformations of the protein species can be compared. The values indicate that the reconstituted proteins are delicately but distinctly different from the native AP. The binding of the apolar dye 1,8-ANS to the proteins indicates that in the refolded species a portion of the hydrophobic patches in the protein structure remained exposed to the polar environment and were accessible to the dye. We also wanted to measure the enzymatic efficiency of the oxidized and reduced forms vis-à-vis the native protein by the usual pNPP assay (Table 2). Results show significant differences in K_M and k_{cat} values for the three forms.

Interaction of GTP with AP. The interaction was monitored by CD spectroscopy in the UV range of 230−300 nm. The region below 230 nm has a dominant CD band due to free GTP which masks the protein bands and is not useful in following the interaction. Figure 5 shows the difference CD spectra of GTP in the presence of native or reconstituted AP. AP concentrations were at a level where no significant CD band due to protein is observed in this region. At 4 mM GTP with reconstituted AP, a strong CD signal in the nucleotide absorption band appears which is not present in the spectrum of free GTP. The same CD bands do appear upon GTP binding to the native AP as well and must be due to induced CD. Overlapping of these spectra suggests that structural elements responsible for the induced CD are present in both the reconstituted AP and the native AP. These bands however are only barely visible in the spectrum of the protein reconstituted with 2 mM GTP, although 60% activity has been regained under this condition. This observation suggests that the binding of GTP at the active site is not responsible for the induced CD signals. The induced signals possibly arise out of binding of GTP to some other site involving asymmetric structural elements, like α-helices, which are fully formed in the native AP as well as in the AP reconstituted with 4 mM GTP. The difference in the CD spectra and enzymatic activity of 2 and 4 mM reconstituted protein can possibly be explained in terms of a dimer assembly process involving GTP binding at the dimerization interface (see the Discussion).

Oxidation of Reduced Reconstituted AP. The 4 mM GTP-refolded AP shows 75−80% of the native activity. It is necessary to see if the reduced AP could be oxidized in vitro to form native S−S bonds with a further increase in the enzymatic activity. Table 3 gives the results of our oxidation experiments and indeed shows significant increases in activity upon formation of S−S bonds. We however want to point out that under our experimental condition the oxidation was incomplete and only a fraction was found to be in the oxidized state when the compound was checked on SDS−PAGE; understandably, the observed increase is not dramatic.

DISCUSSION

Data presented here provide several interesting insights into the GTP-induced refolding of AP. First, there is an inverse relationship between the ability to protect against denaturation and the ability to induce proper refolding (Figures 1 and 2); a good substrate, Gl-6-P, which gives maximum protection to denaturation is a poor inducer of refolding and vice versa. The steric complementarity of the active site for a good substrate is a better match than that for a poor substrate. A good substrate like Gl-6-P sitting tightly at the active site would naturally give maximum protection to denaturation since a large number of binding interactions have to be broken. On the other hand, it is conceivable that initial hydrophobic collapse in the presence of a poor substrate like GTP might lead to generation of an ill-formed active site around the GTP molecule, which may serve as a nucleation center for further refolding. During refolding, steric adjustment necessary for binding GTP will be far less stringent than that required for Gl-6-P binding, and therefore, GTP would be a better inducer than Gl-6-P. We also find that there is practically no difference between the properties of the self-refolded oxidized form (intact S−S bonds) and those of the GTP-refolded reduced form as corroborated by the activity profile (Table 1), I_{330}/I_{350} values, ANS binding, and even the specificity constant K_M/k_{cat} (Table 2). The GTP-refolded reduced form has lower K_M and k_{cat}

Table 2: Comparison of Native and Reconstituted Forms of AP

sample	fluorescence peak (nm)	I_{330}/I_{335}	ANS fluorescence (arbitrary units)	K_M (μM)	k_{cat}[a] (s^{-1})	$k_{cat}/K_M \times 10^{-6}$ ($M^{-1}\ s^{-1}$)
native AP	330	1.43	2.6	71	23	0.32
reconstituted AP without 2-MCE and GTP (oxidized form)	330	1.32	6.0	66	18	0.27
reconstituted AP with 6 mM 2-MCE and 4 mM GTP (reduced form)	330	1.35	6.0	50	14	0.28

[a] The k_{cat} values are calculated from the V_{max} values by using a dimer molecular weight of 94 000.

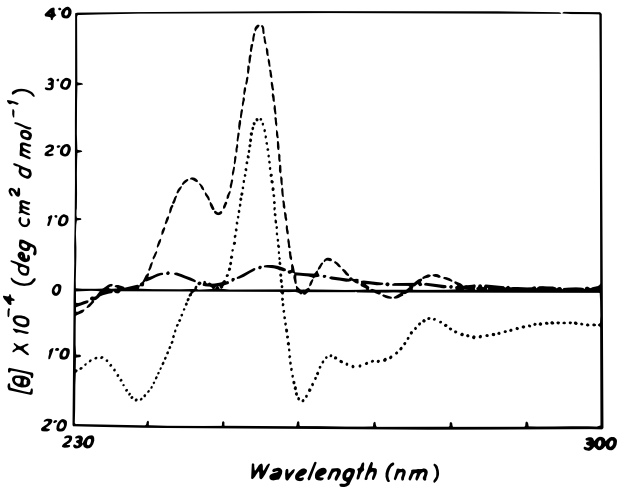

FIGURE 5: Induced CD spectra of bound GTP in the presence of native and reconstituted AP. Spectral data are calculated as (AP plus GTP plus buffer) − (GTP plus buffer). Molar ellipticities are calculated using molar concentrations of GTP: native AP and 4 mM GTP (- - -) and AP reconstituted in the presence of 2 mM GTP (− · −) and 4 mM GTP (···).

Table 3: Enzymatic Activity of Reduced Reconstituted AP upon Treatment with GSSG

AP	activity (% of native) before GSSG treatment	immediately after GSSG addition	after overnight dialysis
4 mM GTP-reconstituted	74.6	76.6	83.5
15 μM Zn^{2+}-reconstituted	51.3	53.6	55.2

values than the self-refolded oxidized form, but the specificity constant, K_M/k_{cat}, which gives a true measure of the efficiency of the enzyme, remains the same for the two refolded forms. Using the relationship described in ref *12*

$$\Delta G_{app} = -RT \ln \frac{(k_{cat}/K_M)_{AP-ox}}{(k_{cat}/K_M)_{AP-red}}$$

the corresponding free energy change is negligible. This suggests that whatever differences exist in substrate binding interactions of the two forms, the important interactions which are used to stabilize the transition state are not sacrificed in either form. This is only possible if the active site architectures in the self-refolded oxidized form and the GTP-refolded reduced forms resemble each other very closely, leading to similarly efficient enzymes, with or without the S−S bonds. We however do not imply that any of the reconstituted forms has a unique structure as in the case of native AP. We think it could be an ensemble of related structures having extremely similar active site architectures which are like native but not quite native structure.

Interestingly, the reduced protein has the lowest K_M, which is substantially lower than that for the native AP, indicating a considerable increase in the affinity for the substrate pNPP. This suggests an increase in the number of binding interactions with the substrate, which is expected from our proposed template type of action of GTP and induced fit, although the binding energy is nonproductive since the k_{cat} also decreased.

We observed that 2 mM GTP and 15 μM Zn^{2+} can restore the activity of AP under reducing conditions up to 60 and 40%, respectively. Interestingly, at 4 mM GTP, the activity went up to 80%, whereas at higher Zn^{2+} concentrations, no such increase in activity was observed. We think Zn^{2+} promotes generation of dimerization-competent monomers through site specific ligand binding; GTP, on the other hand, has a somewhat more prominent role. GTP probably serves as both a substrate and a modulator molecule. Our CD data suggest that GTP binds to another site different than the active site, giving the induced CD bands. Active AP is a dimer with a large subunit interface. The X-ray crystal structure has shown that α-helical regions, which directly follow metal binding histidyl residues from the two subunits, interweave and form the dimerization interface (*13*). Coleman (*3*) suggested that the formation of an active enzyme upon dimerization is a complex process possibly involving some modulator molecules. If we assume that GTP binds in this region of the dimerization interface, this can explain a modulator role of GTP as well as the induction of CD bands. In such a scenario, GTP not only generated dimerization-competent monomers such as Zn^{2+} because of its substrate nature but also possibly assisted (modulated) in forming active dimers to regain 80% of the native activity, somewhat more than that obtained with Zn^{2+}. If we consider a coupled equilibria between unfolded, folded, and Zn^{2+}- or GTP-bound species

$$U_{SH} \rightleftarrows F_{SH} \rightleftarrows F_{SH}(Zn^{2+}/GTP) \rightleftarrows [F_{SH}(Zn^{2+}/GTP)]_2$$

A simple thermodynamic explanation can be offered for reconstitution of active AP under reducing conditions. If the active site specific ligand Zn^{2+} or GTP binds to and stabilizes the properly folded dimerization-competent monomers, that would pull the coupled equilibria to the right. Such a process alone, however, cannot adequately explain the difference in activity of various refolded forms observed in this study. Zn^{2+} (15−20 μM)-assisted refolded AP is only 40% active, which suggests that either population of active

dimers is only 40% or dimerization per se is not enough to gain maximum activity but requires further modulations. Our inability to increase the activity at higher Zn^{2+} concentrations argues against the first possibility. Results from delayed experiments during which GTP (2 mM final concentration) is added to the Zn^{2+}-refolded protein and the activity is raised from 40 to 60% support strongly the second possibility and highlight the importance of GTP in generating active dimers. It may be noted that 2 mM GTP-assisted refolded AP, in the presence or absence of 15 μM Zn^{2+}, shows 60% activity which can be raised to ~80% by delayed addition of more GTP. This result also supports the notion that dimerization of AP is a complex process and possibly requires some modulator molecule (*3*). Our results suggest that GTP not only binds to the active site but also plays the role of a modulator by interacting at the dimerization interface.

ACKNOWLEDGMENT

We thank Dr. Rajendra Prasad (Dean of the School of Life Sciences, Jawaharlal Nehru University) for the use of the CD machine, A. Lynn (SLS) and Tapos Saha (CBT) for CD spectra, and Partha Mukhopadhyay (CBT) for other technical help. We also thank N. S. Kar, Dr. A. Das Gupta, and Prof. M. K. Pal of the Department of Biochemistry and Biophysics (Kalyani University) for helpful discussions. The secretarial help of Ms. G. Khattar is gratefully acknowledged.

REFERENCES

1. Sadis, S., Raghavendra, K., Schuster, T. M., and Hightower, L. E. (1990) in *Current research in protein chemistry: techniques, structure and function* (Villafranca, J. J., Ed.) pp 339−347, Academic Press, New York.
2. Goto, Y., Okamura, N., and Aimoto, S. (1991) *J. Biochem. 109*, 746−750.
3. Coleman, J. E. (1992) *Annu. Rev. Biophys. Biomol. Struct. 21*, 441−483.
4. Kim, E. E., and Wyckoff, H. W. (1989) *Clin. Chim. Acta 186*, 175−188.
5. Akiyama, Y., and Ito, K. (1993) *J. Biol. Chem. 268*, 8146−8150.
6. Derman, A. I., and Beckwith, J. (1991) *J. Bacteriol. 173*, 7719−7722.
7. Derman, A. I., and Beckwith, J. (1995) *J. Bacteriol. 177*, 3764−3770.
8. Schlessinger, N. J., and Barrett, K. (1965) *J. Biol. Chem. 240*, 4284−4292.
9. Reynolds, J. A., and Schlessinger, M. J. (1967) *Biochemistry 6*, 3552−3559.
10. Sone, M., Kishigani, S., Yoshihisa, T., and Ito, K. (1997) *J. Biol. Chem. 272*, 6174−6178.
11. Sarkar, S. N., and Ghosh, N. (1996) *Arch. Biochem. Biophys. 330*, 174−180.
12. Fersht, A. R. (1985) in *Enzyme Structure and Mechanism*, 2nd ed., Chapter 12, W. H. Freeman & Co., New York.
13. Coleman, J. E., and Gettins, P. (1983) *Adv. Enzymol. 55*, 381−452.

BI972833G