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Overexpression and Refolding of the Hydrophobic Ribosomal P0 Protein from *Trypanosoma cruzi:* A Component of the P1/P2/P0 Complex

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The P0 protein is part of the ribosomal eukaryotic stalk, which is an elongated lateral protuberance of the large ribosomal subunit involved in the translocation step of protein synthesis. P0 is the minimal portion of the stalk that is able to support accurate protein synthesis. The P0 C-terminal peptide is highly antigenic and a major target of the antibody response in patients with systemic lupus erythematosus and patients suffering chronic heart disease produced by the *Trypanosoma cruzi* parasite. The *T. cruzi* P0 (TcP0) protein was cloned into the pRSET A vector and expressed in *Escherichia coli* fused to a His-tag. The identity of the protein was confirmed by immunoblotting. Due to the formation of inclusion bodies the protein was purified using the following steps: (i) differential centrifugation to separate the inclusion bodies from soluble proteins and (ii) affinity chromatography under denaturing conditions. TcP0 showed high tendency to aggregation during refolding assays. However, TcP0 could be efficiently folded in the presence of a low concentration of SDS. The folding of the protein was confirmed using urea gradient electrophoresis, limited proteolysis, circular dichroism, and tryptophan fluorescence. Native electrophoresis showed that the folded TcP0 (and not a folding intermediate) was the cause of aggregation in the absence of SDS. The protocol described here permitted us to obtain large amounts (up to 30 mg per culture liter) of pure and folded TcP0, a very hydrophobic protein with a high tendency to aggregation.

The ribosomal stalk is a structural feature present in the large subunit of eukaryotic and prokaryotic ribosomes that is involved in the interaction of elongation factors with the ribosome. Although the structure of the large ribosomal subunit has been described for bacteria at atomic resolution (1) there is not clear electronic density for the ribosomal stalk proteins. Electron microscopy and low-resolution X-ray studies have suggested that the ribosomal stalk is an elongated and highly flexible lateral protuberance on the large ribosomal subunit (2). The prokaryotic stalk is made up of two dimers of the 12-kDa acidic ribosomal proteins L7/L12, forming a very stable complex with protein L10 (3). In eukaryotes this pentamer is formed by the family of ribosomal P proteins, P0, P1, and P2, which show no significant sequence identity with the analogous bacterial proteins L10 and L7/L12.

The ribosomal stalk has been proposed to play a role in the translocation step of protein synthesis (2, 4). Moreover, it has been shown to be involved in the activity of the elongation factors in both bacteria (5) and eukaryotes (6, 7). A direct interaction between the ribosomal stalk and EF-Tu and EF-G has been recently confirmed by electron microscopy (8, 9).

The small P proteins (P1 and P2) interact via their N-terminal domains with a region close to the C-terminus of P0 (10, 11). The C-terminal domains of P1 and P2, on the other hand, are exposed to the medium and

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interact with the elongation factors (8, 9). Several subclasses of small P proteins exist but their occurrence is variable from species to species. In mammals there is only one of each class (P1 and P2) whereas in plants a third type, P3, has been reported. *Saccharomyces cerevisiae* possesses P1α, P2 α, P1β and P2β and *Trypanosoma cruzi* has P1, P2α, and P2β. However, it is apparently necessary to have at least one copy of each of the two types (P1 and P2) in order to form the pentameric complex with P0 (12).

Although the bacterial and eukaryotic stalk proteins seem to play a similar role, there are several important differences. In particular, P0 shows a higher degree of structural and functional complexity than the bacterial homologue L10 (10, 13). The eukaryotic protein has a C-terminal region of around 100 amino acids that resembles the amino acid sequence of P1 and P2 (14). This extension plays an important role in the formation of the pentameric complex with P1 and P2 proteins (10). In addition, P0 is able to perform the functions of the whole stalk in the absence of the other components (15). In conclusion, P0 is the minimal portion of the stalk that is able to support accurate protein synthesis albeit at a lower rate than the complete pentameric complex (16).

P0 also binds to the GTPase site on the 28S rRNA via an RNA-binding domain containing approximately 200 residues that is localized in the N-terminal region of the protein (10). The GTPase site of the rRNA molecule is highly conserved and has been shown to be interchangeable between bacteria and eukaryotes (17, 18). However, the binding of P0 to rRNA is stronger than in the prokaryotic system (19, 20). Besides the RNA-binding domain, P0 possesses two additional but less well-conserved functional domains, (i) the P1/P2-binding domain and (ii) the elongation-factors-binding domain. Both domains of the latter are probably located in the C-terminal region.

P0 has in common with P1/P2 a highly conserved C-terminal peptide, which is connected to the rest of P0 by an alanine-rich tract (14). This region of P0 is essential for activity and probably plays a central role in the interaction with the soluble factors of the cytoplasm (10). The C-terminal peptide of P0 and the corresponding regions of P1/P2 are highly antigenic and are the major target of the antibody response in 10–15% of patients with systemic lupus erythematosus (21). Antibodies against this region of the P proteins have also been detected in patients with chronic Chagas disease (22) and leishmaniasis (23). Furthermore, it has recently been reported that antibodies against the N-terminal region of P0 inhibit the growth of *Plasmodium falciparum in vitro* (24). As a consequence of the many clinical implications of these proteins in different diseases, there is a considerable amount of information concerning their immunological, biochemical, and functional properties. In contrast, however, there is relatively little data available concerning their three-dimensional structure and folding properties, particularly in the case of P0.

The understanding of the folding pathways of a protein is based on the identification of the possible folding intermediates existent along each route, i.e., an examination of the conformations which exists between the fully unfolded state and the condensed folded state (25, 26). While both fully denatured and folded native states are significantly populated at equilibrium, partially folded intermediates are frequently unstable and are not present in significant concentrations, because the folding is a highly cooperative process. Consequently, they are difficult to detect and characterize. These intermediate states are very important because they are the major cause of aggregation that leads to low yields in the *in vitro* refolding protocols of many proteins.

Transverse urea gradient electrophoresis is a simple and useful tool to analyze the folding/unfolding processes of proteins (27,28). The protein is subject to electrophoresis in an acrylamide gel containing a concentration gradient of urea perpendicular to the direction of migration. Urea-induced conformational transitions that are accompanied by alterations in shape are detected as changes in the electrophoretic mobility due to the larger hydrodynamic volume of the unfolded protein with respect to the native form. Relatively long-lived folding intermediates along the unfolding or refolding pathways would be trapped and detected as bands with intermediate electrophoretic mobility.

In the present work we describe the expression, purification, and initial structural and folding–refolding studies of the *T. cruzi* ribosomal P0 protein (TcP0). The final objective of this work is to obtain adequate amounts of this protein to use in functional and structural studies.

METHODS

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)[2] *and Immunoblotting*

Anti-sera against TcP0 were obtained by immunizing Balb/c mice with a protein made of TcP0 (amino acids 32–323) fused to maltose-binding protein.

Twelve percent SDS–PAGE under reducing conditions was performed using a standard procedure (29). The proteins were transferred to nitrocellulose membranes and blocked overnight at 4°C with 3% nonfat milk in Tris-buffered saline (TBS-M). Mouse anti-serum

[2] Abbreviations used: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-thiogalactoside.

against TcP0 diluted 1:200 in TBS-M was used as first antibody. The background was reduced adding 10% of *Escherichia coli* lysate nonexpressing TcP0. The first antibody was detected with anti mouse IgG biotinylated and developed using the ABC kit (Vector Laboratories Inc., Burlingame, CA).

Obtention of Recombinant Plasmids

Recombinant DNA manipulation followed standard procedures (30). The cDNA for TcP0 (GenBank Accession No. X6506) was cut from a plasmid previously obtained in our laboratory (pcDNA3–TcP0, unpublished results) and subcloned in the pRSET A expression vector.

Briefly, the cDNA corresponding to TcP0 was obtained by PCR on cDNA from *T. cruzi* (Tulahuen strain). The primers were designed to add 5′ *Bam*HI (5′ GGGGATCCAAAATGCCGTCTGTCTCC 3′) and 3′ *Xba*I (5′ CCTCTAGATCAAAACAGCGCCCCCATG 3′) restriction sites (underlined) to the TcP0 cDNA. The amplification product was then subcloned in the *Bam*HI and *Xba*I sites from the pcDNA3 vector (Invitrogen). The insert was sequenced to confirm the authenticity of the recombinant clone (pcDNA3-TcP0).

TcP0 cDNA was cut from pcDNA3–TcP0 with *Bam*HI and *Sac*I (pcDNA3 have a *Sac*I site 40 bp downstream from the polylinker) and subcloned into the *Bam*HI and *Sac*I sites of the expression vector pRSET A (Invitrogen) to obtain the recombinant plasmid pRSET A–TcP0 (Fig. 1).

To obtain a vector expressing TcP0 without any tag, the fusion-peptide-coding region from pRSET A–TcP0 was removed using restriction enzymes. pRSET A–TcP0 was digested separately with *Bam*HI (aliquot 1) and *Nde*I (aliquot 2), because TcP0 cDNA has an internal *Nde*I restriction site (see Fig. 1). The protruding ends of both aliquots were filled with Klenow DNA polymerase (Promega). Both aliquots were cut with *Sac*I and the DNA fragments corresponding to cDNA TcP0 (aliquot 1) and plasmid (aliquot 2) purified and ligated using T4 DNA ligase. The absence of the fusion-coding region was confirmed by the inability of *Nhe*I (one *Nhe*I site is present in the fusion-coding region of pRSET A, see Fig. 1) to cut the plasmid. Remarkably, this procedure eliminates the fusion-coding region but not the ribosome binding site, which is located upstream from the *Nde*I site in pRSET A–TcP0.

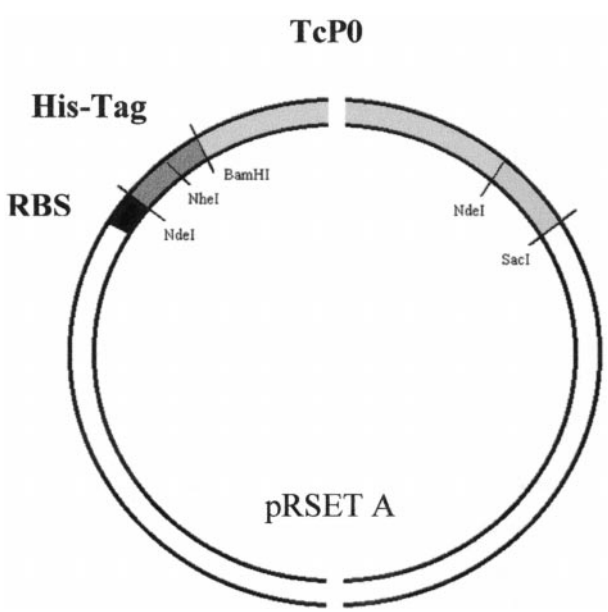

FIG. 1. pRSET A–TcP0. Representation of the plasmid pRSET A–TcP0 used for the expression of TcP0 fused to a N-terminal His-tag. Only relevant restriction sites are shown. RBS, ribosome-binding site.

Expression and Purification of the Recombinant TcP0

pRSET A–TcP0 was transferred to *E. coli* BL21-(DE3)pLysS-competent cells (Invitrogen). Colonies from this transformation were grown at 37°C up to an optical density of 0.6 at 600 nm. The cultures were induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and grown for an additional 2 h at the same temperature. The bacteria were recovered by centrifugation, resuspended in native buffer (Na_2HPO_4 50 mM, NaCl 300 mM, pH 8), and lysed by freezing–defrosting cycles and sonication.

To check the efficiency of the lysis, a small aliquot of the lysate was centrifuged at high speed (12,000*g*, 30 min). The pellet and the supernatant were analyzed by SDS–PAGE and stained with Coomassie blue. The observation of bands that are only present in the supernatant (soluble proteins) indicates that the lysis was complete and the protocol can be continued.

The lysate was centrifuged at 1000*g* for 20 min and the pellet was resuspended in 10 vol of native buffer, washed, and centrifuged twice. The final pellet was resuspended in 10 vol of buffer A (8 M urea, 10 mM Tris, 100 mM Na_2HPO_4, 10 mM 2-mercaptoethanol, pH 8), left for 1 h at room temperature and finally centrifuged for 1 h at 10,000*g*. The supernatant was mixed with the Ni–NTA resin (Qiagen), which had been previously equilibrated with buffer A (6 mg or more of TcP0 was added for each ml of resin). The mixture was incubated with slow agitation for 1 h at room temperature and poured into the column. The flowthrough was collected and the column subsequently washed four times with 3 vol of buffer A followed by four washes with 3 vol of buffer B (8 M urea, 10 mM Tris, 100 mM Na_2HPO_4, 10 mM 2-mercaptoethanol, pH 6.3). The protein was eluted sequentially with buffer C (8 M urea, 10 mM Tris, 100 mM Na_2HPO_4, 10 mM 2-mercaptoethanol, pH 5.9) and buffer D (8 M urea, 10 mM Tris, 100 mM Na_2HPO_4, 10 mM 2-mercaptoethanol, pH 4.5). Ten

fractions (1 column vol per fraction) were collected with each buffer. The eluted fractions were analyzed by 12% SDS–PAGE. The protein concentration was estimated by the method of Bradford (31) using bovine serum albumin as standard.

PAGE with a Discontinuous Transverse Urea Gradient

Urea gradient gels were prepared by stacking nine layers of acrylamide solutions of increasing urea concentration, ranging from 0 to 8 M, in steps of 1 M. The linear urea gradient was superimposed on an inverse gradient of 9 to 6% acrylamide to compensate for the viscosity change produced by the urea. The buffer used throughout the gel was 0.3 M Tris–HCl, pH 8.8. The stacking gel was prepared with 4% acrylamide and 0.3 M Tris–HCl, pH 8.8. When unfolded protein was analyzed with this method, 8 M urea was added to the stacking gel. The electrophoresis buffer used was 0.1 M Tris, 0.384 M glycine (pH 8.3–8.5). Electrophoresis was performed at 20 mA at room temperature using a Mini-Protean II cell (Bio–Rad). The protein was detected by staining with Coomassie blue. Where indicated, SDS was added to the samples and both the electrophoresis and gel buffers at a concentration of 0.01% (w/v).

TcP0 Refolding

One volume of TcP0 in elution buffer (C or D) was fractionated in ten aliquots and added sequentially at intervals of 20 min, with stirring, to 50 vol of refolding buffer (0.3 M Tris–HCl, pH 8.8–0.01% SDS (w/v)–10% glycerol (v/v)) at a temperature of 4°C. The final protein concentration was 50 μg/ml. The sample was left overnight at 4°C and then concentrated to the original volume by filtration using Centricon tubes (Amicon) with a 10-kDa cutoff.

The samples used for spectral studies (circular dichroism and tryptophan fluorescence) were prepared using 50 mM Na_2HPO_4/NaH_2PO_4, pH 8, 0.01% SDS as the refolding buffer, to avoid the interference of Tris in the low-wavelength region (circular dichroism). The absence of aggregation was confirmed by native PAGE.

Limited Proteolysis

Thirty micrograms of unfolded TcP0 (in 0.3 M Tris–HCl, pH 8.8; 0.01% SDS; 10% glycerol; 4 M urea) or folded TcP0 (in 0.3 M Tris–HCl, pH 8.8; 0.01% SDS; 10% glycerol) were incubated for 10 min with 5 μg of porcine trypsin (Sigma) in a final volume of 200 μl at 20°C. The reaction was stopped by the addition of 1 vol of SDS–PAGE sample buffer 2× and boiling it for 4 min. The proteolysis products were run in a 17% SDS–PAGE and stained with Coomassie brilliant blue.

Spectroscopic Analysis

The protein concentration was determined from the absorbance at 280 nm using an extinction coefficient of 23,200 M^{-1} cm^{-1}, calculated from the amino acid composition of the protein.

CD spectra were obtained with a Jasco J-710 spectropolarimeter, using a 0.1-cm pathlength quartz cuvette. Spectra were the average of three scans at 50 nm/min speed with a response time of 4 s at 20°C. The protein concentration was 0.3 mg/ml. Helix content was estimated from the CD signal by dividing the mean residue ellipticity at 222 nm by the value expected for 100% helix formation, $-33{,}000$ deg cm^2 $dmol^{-1}$ (32).

Urea equilibrium denaturation was carried out in 50 mM Na_2HPO_4/NaH_2PO_4, pH 8, 0.01% SDS at increasing urea concentration, ranging from 0 to 8 M. The temperature was fixed at 20°C. The protein concentration was 0.2 mg/ml. Fluorescence spectra were obtained using an Aminco Bowman spectrofluorometer. The excitation wavelength was 290 nm and the emission was scanned from 300 to 400 nm. The urea denaturation curve was made plotting the emission intensity at 320 nm versus the urea concentration. The data was adjusted to a two-state model using minimal squares.

RESULTS

Expression of Recombinant TcP0

TcP0 was expressed as described above. The protein expression level was high (>50 mg/liter of culture) but it was in the form of inclusion bodies and the protein could not be detected in the soluble fraction. In good correlation with the theoretical molecular weight (39.215 kDa) the fusion product migrates as a band of around 40 kDa on SDS–PAGE. Figure 2A shows the TcP0 expression at different induction times employing 0.1 mM IPTG. Specific antibodies (Fig. 2B) recognized the TcP0 protein band.

TcP0 was also expressed without the tag using a modified vector obtained by the elimination of the fusion-peptide-coding region in the pRSET A–TcP0 vector (see Methods). The expression level was very low suggesting that the fusion peptide (probably the T7 gene 10 leader) is important for the expression of TcP0 (data not shown).

Purification of TcP0

The first step was a differential centrifugation to separate the inclusion bodies from the lysate. This step was very effective in the isolation of TcP0 (see Fig. 3B). The washed pellet was dissolved in denaturing buffer and purified by affinity chromatography as described under Methods. To increase the purification efficiency the protein was added in excess with respect to the resin. This led to saturation of the resin binding sites

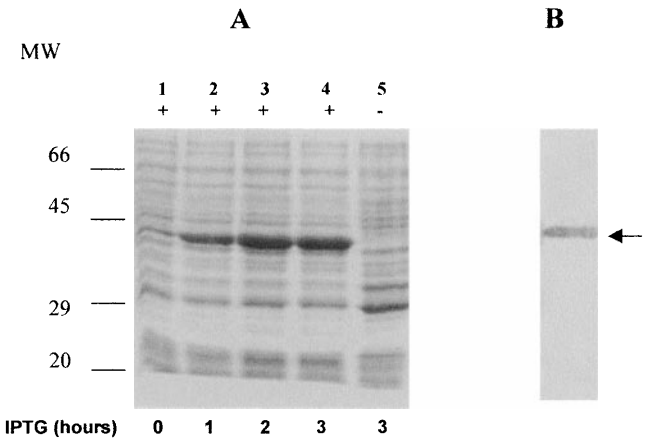

FIG. 2. Expression of recombinantTcP0 and its identification by specific antibodies. (A) Cultures of colonies transformed with pRSET A-TcP0 (+) or pRSET A (−) were grown up and induced with IPTG. The induction times are shown below. The samples were run in 12% SDS–PAGE and revealed with Coomassie Blue. (B) A sample identical to lane 4 of A was run, transferred to nitrocellulose membranes, and revealed with mouse antiserum against TcP0. The arrow on the right shows the migration position of TcP0.

with the protein of interest thus avoiding the binding of nonspecific proteins. With this procedure it was possible to obtain 2–3 mg of pure protein for each milliliter of resin and 20–30 mg per culture liter. Figure 3 shows the SDS–PAGE of aliquots taken during the three purification steps. Table 1 shows the fraction of total protein corresponding to TcP0 at each purification stage.

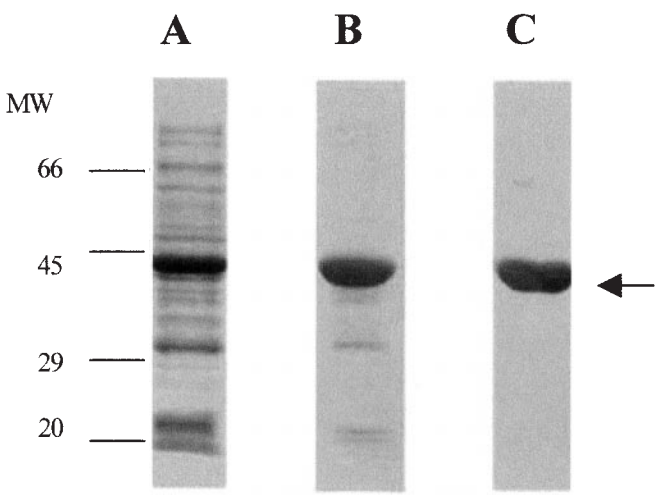

FIG. 3. Purification of recombinant TcP0. 12% SDS–PAGE of samples at different stages of purification were stained with Coomassie blue. (A) Crude extract of cultures expressing TcP0. (B) Inclusion bodies containing TcP0 washed extensively with native buffer by differential centrifugation as described under Methods. (C) Aliquot of the eluted fractions from the affinity column under denaturant conditions. Note the great enrichment of the extract in TcP0 after washing the inclusion bodies. The arrow on the right shows the migration position of TcP0.

TABLE 1

Purification Scheme

Purification step	% of total protein[a]	mg of TcPO
Total lysate	28	>50
Washed pellet	62	>50
Affinity purification	>99.5	up to 30[b]

Note. The data were obtained from 1 liter of culture. Experimental conditions were as described under Methods.

[a] The ratio of TcPO to total protein was determined by gel scanning.

[b] The limitant condition in this step was the amount of affinity resin used. Approximately 2.5–3 mg per ml of resin and up to 30 mg per liter of culture can be obtained.

Refolding of TcP0

Denatured TcP0 was subject to urea gradient electrophoresis and the gel was stained with Coomassie blue (Fig. 4A). Refolding could not be detected at low urea concentration because the protein is totally aggregated. There are basically two possible causes that could lead to this aggregation process:

(i) The folded state of TcP0 could be inherently unstable and have a great tendency towards aggregation. P0 proteins are located in the ribosome where they interact with other ribosomal components, which probably lead to the occlusion of hydrophobic surface patches on P0 from the aqueous environment.

(ii) The cause of aggregation could be the result of intermediates along the folding pathway. This phenomenon has been observed in the folding of many proteins.

It was necessary to elucidate which of these phenomena is the real cause of aggregation in order to design an appropriate refolding strategy for the production of large amounts of TcP0 for structural analysis.

When the urea gradient electrophoresis was performed in the presence of 0.01% SDS (Fig. 4B) a typical folding curve was clearly visible and the aggregation decreased drastically. This result was useful in establishing the conditions necessary for the adequate *in vitro* refolding of TcP0 as described under Methods. Neutral detergents such as Triton X-100, NP-40, and Tween 20 were not effective in preventing aggregation.

To verify the success of refolding the renatured and concentrated sample was also run on a urea gradient gel. Figure 4C showed that there was not sign of aggregation of TcP0 in the zones of the gel corresponding to low urea concentration, indicating that the renaturation was complete. Furthermore, a classical cooperative unfolding curve was observed for TcP0. From the comparison of Figures 4B and 4C some differences between folding and unfolding patterns could be observed. In the case of the folding curve (Fig. 4B) the protein band in the transition zone and the zone corresponding to the folded protein are both diffuse. In the unfolding

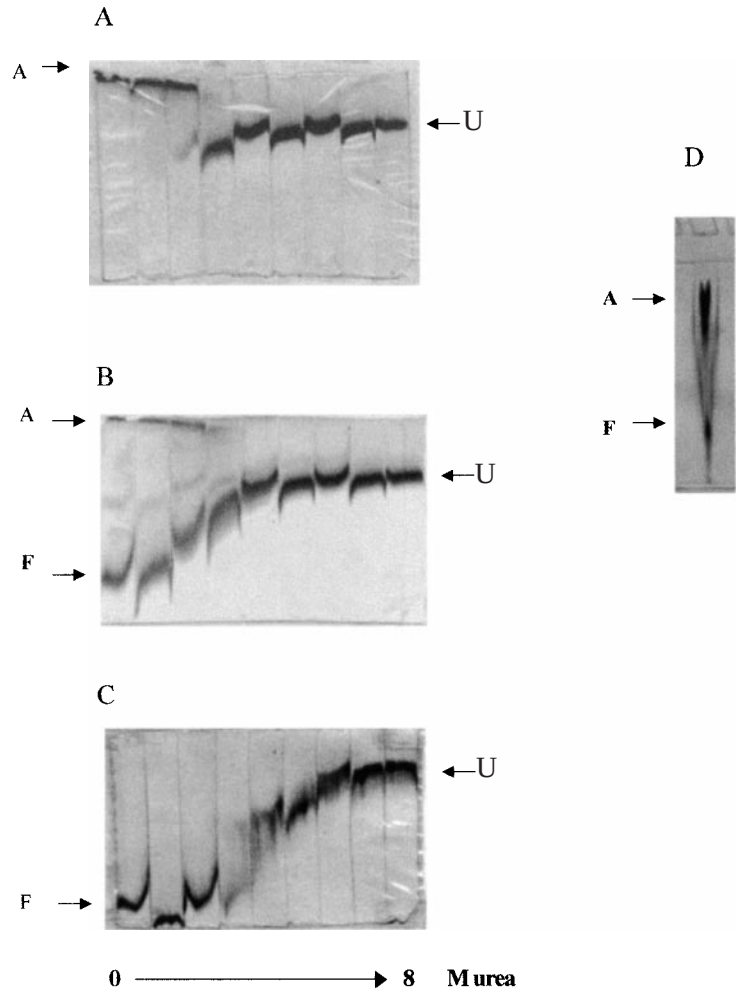

FIG. 4. Aggregation of folded TcP0 is prevented by low SDS concentration. (A) TcP0 purified under denaturant conditions was subject to electrophoresis through a linear gradient of urea. It showed only aggregation phenomenon at low urea concentration. B. Unfolded TcP0 was electrophoresed as in A but with the addition of 0.01% SDS as described under Methods. Note that in the presence of SDS the folded monomer is the predominant specie at low urea concentration. (C) *In vitro* refolded TcP0 was subject to urea gradient electrophoresis as in B. Note the unfolding curve and the total absence of aggregation at low urea concentration. (D) Refolded and concentrated TcP0 was subject to native electrophoresis. The conditions were identical to those in urea gradient gels but without the addition of either SDS or urea. Note the aggregation of the folded protein in the absence of the detergent. The gels were stained with Coomassie blue. The arrows indicate folded (F), unfolded (U), and aggregated (A) protein.

curve (Fig. 4C) the protein band becomes diffuse in the transition zone and in the zone of the unfolded protein. These observations suggest that the folding/unfolding transition is slow in the time scale of the electrophoresis.

The patterns of TcP0 in urea gradient gels do not showed evidence of the presence of intermediate states during unfolding/folding transitions. These observations could be explained by a simple folding model with only two states (folded and unfolded) present in significant concentration. This model implies that the state causing the aggregation of TcP0 is the fully folded protein.

However, we can not rule out the presence of intermediate states, which may aggregate as soon as they form and could not be detected by the urea gradient electrophoresis technique.

To confirm the hypothesis that the folded TcP0 was responsible for the aggregation refolded TcP0 was run in a gel under the same conditions used before without the addition of SDS and urea. Figure 4D shows that refolded TcP0 was aggregated in the absence of SDS. A small fraction of the protein migrates as a monomer. This is caused by the small amount of SDS contained in the sample.

To confirm the refolding success limited proteolysis assays were made on folded and unfolded TcP0. Figure 5 shows clearly that the folded protein was much more resistant to proteolysis than the unfolded one.

Due to the difficulty to obtain P0 proteins in the pure

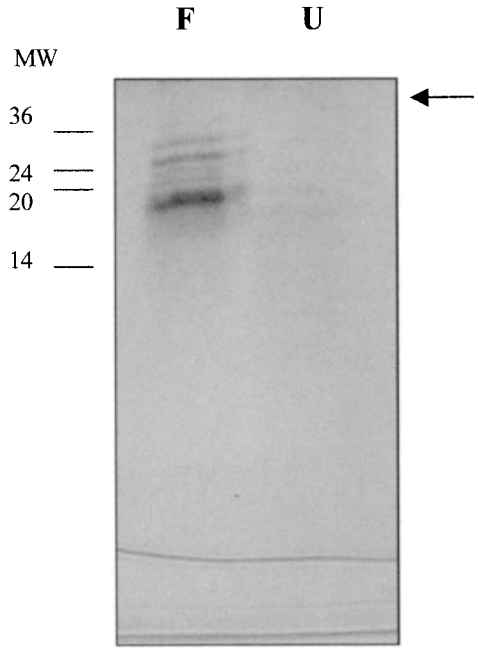

FIG. 5. Limited proteolysis of folded and unfolded TcP0. Tryptic digestions on folded (F) and unfolded (U) TcP0 were carried out as described under Methods. The reaction was stopped and the samples were resolved by 17% SDS–PAGE. The gel was stained with Coomassie brilliant blue. Note the very low susceptibility to digestion of the folded TcP0. The arrow on the right shows the migration position of undigested TcP0.

and folded state most of the structural and functional information about these proteins has been obtained indirectly; principally through the use of diverse genetic constructions in yeast. The interaction between the P1/P2/P0 complex and 28S rRNA has been studied directly, in isolate form, only for the rat complex (33). In this work, it is shown that the complex can be assembled *in vitro* starting from a mix containing purified P1, P2, and P0 proteins and 28S rRNA in the presence of 6 M urea. The complex is obtained by dialysis against native buffer. However, in the absence of the P1 and P2 proteins, P0 protein aggregates, in accordance with the results showed in our work.

In the *T. cruzi* system, the influence of P1/P2α/P2β and posttranslational modifications (like phosphorylation) on the binding of TcP0 to 28S rRNA is unknown. Consequently, a similar functional test with the purified and refolded TcP0 protein is not possible yet. P1, P2α, and P2β proteins from *T. cruzi* are being subcloned in our laboratory to assemble the P0/P1/P2 complex *in vitro* for future studies.

Next to biological function, the most powerful methods applied as conformational probes, are the spectral methods like circular dichroism and tryptophan fluorescence.

Circular dichroism is a very useful tool to analyze the secondary structure of proteins. CD spectra were made on refolded TcP0. These spectra showed that TcP0 has an important content of helix structure, indicative of a native conformation, as can be inferred from prominent negative peaks at 208 and 222 nm and a positive peak around 190 nm (Fig. 6A). The mean residue ellipticity at 222 nm permits to estimate a helix content of 26%, approximately. The cooperative unfolding of a protein is a good indicator of the presence of tertiary structure. In tryptophan fluorescence studies TcP0 showed a cooperative unfolding (Fig. 6B). These facts, together with the evidence shown by the urea gradient gels and the limited proteolysis assays, strongly suggest the presence of a folded, native conformation of TcP0.

DISCUSSION

TcP0 shows a great tendency towards aggregation. Attempts of renaturation of TcP0, rapidly (by dilution) or slowly (by dialysis), from inclusion bodies solubilized in 8 M urea were unsuccessful. Aggregation is total even if the process is performed at high salt concentration, in the presence of up to 10% glycerol and a at very low concentrations of protein (<5 μg/ml), conditions that are normally used to minimize aggregation.

Urea gradient electrophoresis of TcP0 was performed to have a better understanding of the aggregation process. The analysis of the gel indicates that at low urea concentrations the protein is totally aggregated. Because of the highly hydrophobic character of TcP0, we postulate that probably the folded, native conformation of the protein, rather than a folding intermediate, may be responsible for aggregation.

If this hypothesis is correct the stabilization of the monomeric folded state should prevent aggregation during refolding. Several detergents were assayed in order to test this hypothesis. SDS showed to be the most effective. In the presence of this detergent a folding pattern was clearly visible with a notable reduction in aggregation. This observation suggests that SDS at 0.01% concentration prevents the aggregation of the folded protein without affecting the folding/unfolding equilibrium, in accordance with the model shown in Fig. 7.

The following conditions: 0.3 M Tris–HCl, pH 8.8, 0.01% SDS, 10% glycerol at a temperature of 4°C were used to perform the refolding of TcP0 by *in vitro* dilution. The refolded protein was used to carry out additional urea gradient electrophoresis to check the absence of aggregation and to compare the patterns of folding and unfolding. These assays indicated that the transition is slow with respect to the time scale of the electrophoresis. The success of refolding was also confirmed using limited proteolysis (Fig. 5), circular dichroism (Fig. 6A), and urea equilibrium unfolding followed by tryptophan fluorescence (Fig. 6B).

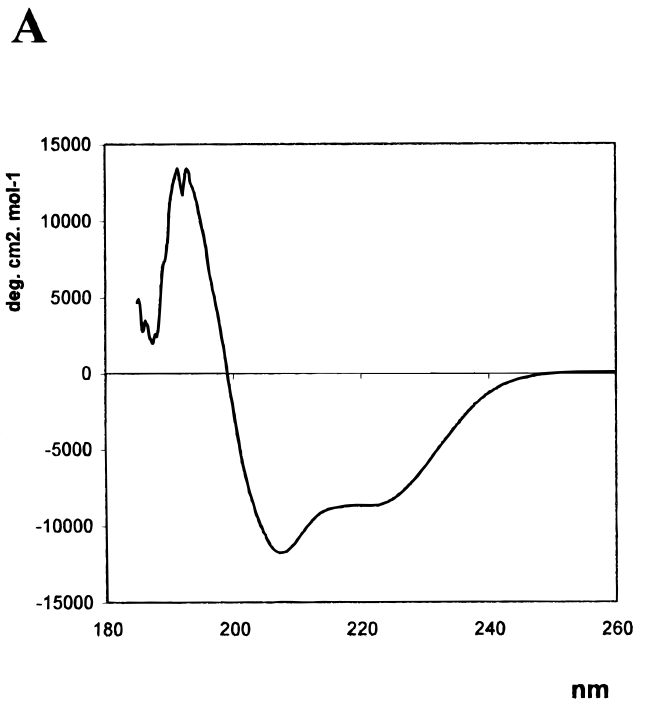

FIG. 6. Spectroscopic analysis of TcP0. (A) CD spectra of *in vitro* refolded TcP0 shows a characteristic double minima at 208 and 222 nm implying an important helix content (26%). (B) Cooperative urea-induced unfolding of TcP0 followed by the intensity of fluorescent emission at 320 nm.

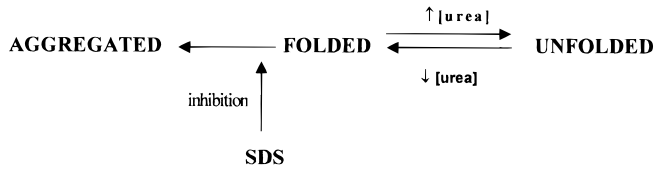

FIG. 7. Folding models suggested by the patterns in native and urea gradient electrophoresis. A reversible equilibrium could exist between the folded and unfolded states. However, the folded form of TcP0 is aggregated in an irreversible way. The aggregation process is avoided by the addition of SDS.

Although the influence of posttranslational modifications (like phosphorylation) on the solubility and stability of TcP0 has not been evaluated, the results presented here may provide a simple explanation to the fact that P0 proteins do not occur freely in the cytoplasm (34). Furthermore, we have described a simple method to obtain large amounts of pure TcP0 and a refolding protocol with a very high level of efficiency. The protocol described here allows the production of protein in adequate amounts to perform spectroscopic and crystallographic structural studies. Finally, the present work confirms once again the utility of the urea gradient electrophoresis technique for the qualitative analysis of folding/unfolding transitions and the rational design of refolding strategies.

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