Biochemical and Biophysical Research Communications **262,** 127–131 (1999)
Article ID bbrc.1999.1107, available online at http://www.idealibrary.com on IDEAL®

Properties of Thiol-Specific Anti-oxidant Protein or Calpromotin in Solution

Poul Kristensen, Daniel E. Rasmussen, and Berit I. Kristensen[1]
The August Krogh Institute, University of Copenhagen, Denmark

Received June 29, 1999

We have isolated calpromotin, a protein reported abundant in human red cells and shown to be of significance for KCl transport. We show that calpromotin is identical to a radical scavenger protein, thiol-specific antioxidant protein (TSA). Calpromotin is known to exist partially as a large complex of identical subunits and partially as dimers probably held together by disulfide bridges. The stability of the high-molecular-weight form was studied by variations of pH and urea concentration. It is shown that the equilibrium between the large complex and the dimeric subunit is governed by the dissociation of a group with a p*K* value of about 7.5. Dissociation of the complex was also complete at 2.5 M urea, where no unfolding of the peptide chains was detectable. © 1999 Academic Press

The role of thiol groups in biological processes has most often been disclosed by observing effects of the classical reactants iodoacetate, iodoacetamide, or *N*-ethylmaleimide. In the case of membrane transport processes, such experiments have led to rather complicated discussions (1), and it is still far from clear, whether the effects are due to direct interactions of the agents with thiol groups present in the membrane proteins responsible for transport, or if the effects are secondary and of a more general nature. In a study of the calcium activated potassium channel in human red blood cells (2, 3) a 23-kDa protein was found to have stimulatory effect. This protein, named calpromotin, was shown to exist both as a high molecular weight aggregate or multimer ($M_r > 300$ kDa) and a low molecular weight form that probably consists of two identical polypeptide chains of 23 kDa (4). Estimation of the number of thiol groups (4) under various conditions showed that the native protein did not react with DTNB (Ellman's reagent, 5,5′-dithio(bis-2-nitrobenzoic acid)), but did so after additon of SDS. After treatment with SDS and DTT (dithiothreitol) simultaneously, three thiol groups were found per monomer. This rather complicated behavior, both with respect to aggregation and with respect to oxidative status or reactivity of the thiol groups, encouraged us to obtain more information on the variables that govern the association of the dimers to form multimers, as this would be advantageous in planning experiments involving the complicated interplay between ion transport processes, e.g., the calcium activated potassium channel, and the various possible regulatory proteins like calmodulin, annexins and calpromotin. We therefore isolated calpromotin and studied the stability of the high molecular weight aggregate by variation of pH and urea concentrations and found that the aggregate can be dissociated by both high pH (>7.8) and by urea concentrations higher than 2.5 M. During our study we also sequenced a peptide obtained by tryptic hydrolysis, and we found that calpromotin is most likely identical to a protein identified by its protective action against free radical damage (5): thiol-specific antioxidant protein (TSA) or thioredoxin peroxidase.

MATERIALS AND METHODS

Isolation of calpromotin from human erythrocytes. Erythrocytes from outdated bank blood was washed four times with PBS (150 mM NaCl, 5 mM sodium phosphate, pH 8.0) with 10 min centrifugation at 8000*g* (av) in a Centricon T-124 high-speed refrigerated centrifuge equipped with an A 8.20 rotor. The packed cells were hemolyzed in 10 vol of buffer A (20 mM Tris, 0.2 mM EDTA, 0.5 mM dithiothreitol, pH 8.0). The membranes were removed by centrifugation at 25,000*g* for 30 min. The hemolysate was stored at −20°C until fractionation. Between 75 and 100 ml of hemolysate were loaded onto a DEAE Sepharose column (1 × 15 cm) equilibrated with buffer A and eluted with a linear gradient of NaCl in buffer A with a final concentration of 0.5 M. Fractions were analyzed by SDS–PAGE and those exhibiting a band with a molecular weight of 22 kDa were pooled and subjected to hydrophobic interaction chromatography on phenyl-Sepharose CL-4B (Pharmacia Biotech). The column (1 × 15 cm) was equilibrated with 0.8 M $(NH_4)_2SO_4$ in 20 mM Tris, pH 8.0, and eluted with a linearly decreasing gradient of the salt. Fractions containing the almost pure 22-kDa protein were pooled, extensively dialyzed against water and finally lyophilized and stored at −20°C. In some cases further purification was obtained by gel filtration on Sepharose S-200 (Pharmacia Biotech).

[1] To whom correspondence should be addressed at The August Krogh Institute, Universitetsparken 13, DK-2100 Copenhagen Ø, Denmark. E-mail: PKristensen@aki.ku.dk.

0006-291X/99 $30.00
Copyright © 1999 by Academic Press
All rights of reproduction in any form reserved.

Unfolding was monitored by the decrease in UV absorption at 286 nm in a series of urea concentrations (0–8 M) in 25 mM K-phosphate buffer, pH 7.0, with and without dithiothreitol (1 mM). The total absorption spectra were recorded in microcuvettes (125 μl) in solutions containing about 1 mg protein per milliliter. The concentration of protein was estimated from the absorbance at 278 nm and an estimated value of ε = 20,700, which is considered sufficient for the present purpose of determining the concentration of urea that was sufficient to dissociate the high molecular weight form without causing unfolding of the monomers.

Analysis of interaction of monomers by gel filtration was performed on a TSK G3000 SW column attached to an Applied Biosystems HPLC system, with a flow rate of 0.5 ml/min. In all cases, the column was equilibrated with at least 2 vol of the eluant before the actual experiment. Protein was monitored at 280 nm. In the urea dissociation experiments the buffer was 50 mM imidazole, 50 mM sodium acetate, 150 mM Na_2SO_4, pH 7.0, +urea at different concentrations. A stock solution of protein (0.4 mg/ml) was diluted about one hour prior to chromatography to 17 μg/ml to obtain urea solutions of the concentrations indicated in the figures. The application volume was 100 μl. The pH dissociation experiments were performed after the same protocol, but with different pH values instead of urea concentrations.

SDS–polyacrylamide gel electrophoresis was carried out exactly as described (6), and the gels were stained with GelCode Blue Stain reagent (Pierce) or Coomassie blue.

Estimation of thiol groups was performed with 5,5′-dithiobis-(nitrobenzoic acid) (DTNB) (5) and results were calculated on the basis of ε_{412} = 13,400 M^{-1} for the nitrobenzoic acid formed. The concentration of protein was estimated from A_{280} and ε_{280} = 20,700 M^{-1}. When measurements were made after reduction with DTT, the latter was removed on a gel filtration column (PD-10, Pharmacia Biotech) that had previously been equilibrated with phosphate buffer at pH 7.2 (with or without 7 M urea) deoxygenized by bubbling with nitrogen for 20 min.

Digestion and sequencing was performed according to standard recipes: About 30 μg of protein was digested over night at 37°C with 2 μg TPCK-treated sequencing grade trypsin (Promega, WI) in 200 μl of 0.1 M Tris/HCl, 10 mM $CaCl_2$, pH 8.5. After acidification with trifluoroacetic acid, the peptides were resolved on a C-18 column (Vydac, 4.6 × 250 mm) with a gradient of acetonitrile in 0.06% trifluoroacetic acid (8).

RESULTS

The purification of calpromotin is summarized in Figs. 1 and 2. The result of the ion-exchange chromatography is almost identical to the earlier report (9), calpromotin being eluted at NaCl concentrations of about 0.2 M. In the next step we used hydrophobic interaction chromatography (phenyl-Sepharose) instead of hydroxyapatite. The first calpromotin containing fractions also contain contaminations of hemoglobin and proteins with molecular weights in the 30-kDa region. The later fractions used for further study did not contain impurities that were detectable with the staining method used. Most important for a comparison with the original preparation (4) we also observed that the protein during gel filtration chromatography was eluted at two positions corresponding to molecular weights of about 300 and 50 kDa. The ultimate test of identity would of course be the presence of identical amino acid sequences. We therefore determined the sequence of a single peptide from a tryptic hydrolysate.

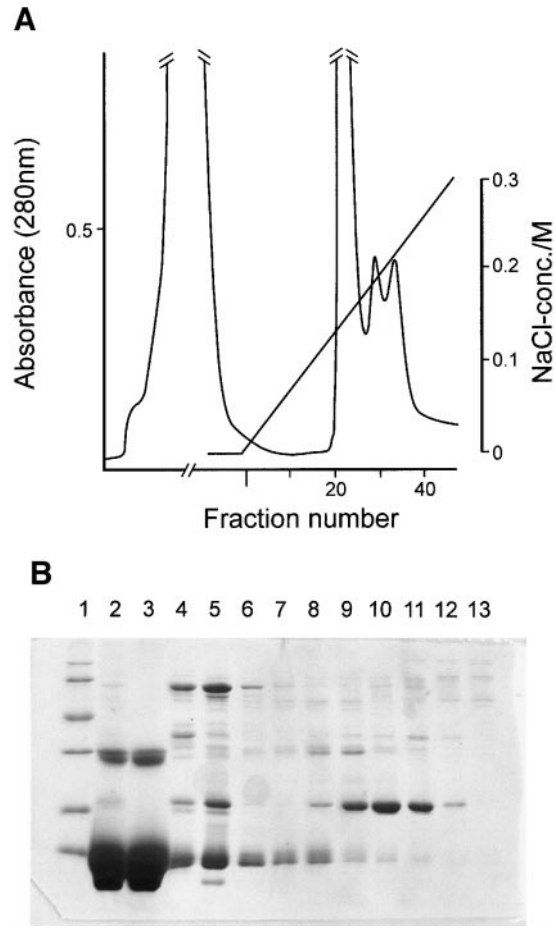

FIG. 1. (A) DEAE-Sepharose anion-exchange chromatography of human red blood cell (HRBC) hemolysate. Hemolysate in buffer A (20 mM Tris, 0.2 mM EDTA, 0.5 mM DTT, pH 8.0) was applied to the column and the column was washed with buffer A till the absorbance at 280 nm was negligible. Elution was performed with a linear gradient of NaCl (0.5 M NaCl in buffer A). Fractions of 2 ml were collected. Fractions 31–38 were pooled for further purification. (B) SDS–polyacrylamide gel electrophoresis in a 12% mini-slab gel of fractions from DEAE Sepharose chromatography of human red cell hemolysate. Molecular weight standards (lane 1), 94, 67, 45, 31, 21, and 14 kDa; lane 2, HRBC hemolysate; lane 3, flowthrough; lanes 4–13, fractions 22, 24, 26, 28, 30, 32, 34, 36, 38 and 40.

The sequence found was YVVLFFYPLDFTFV. It turned out that sequence data for calpromotin was not available, but a database search showed that a single protein contained an identical sequence: Thiol-specific anti-oxidant protein (TSA, Swiss-Prot ID: P32119, AC: TDX1_human).

As with TSA, calpromotin contains three cysteines per polypeptide chain (4). We measured the content of thiols in our preparation of calpromotin under different conditions. The isolated product showed virtually no reactivity, but denaturation with 7 M urea at pH 7.2 (0.1 M sodium phosphate) increased its reactivity to 0.63 ± 0.01 thiols per monomer. If the native protein was treated with DTT, the content of thiols was 1.7 ±

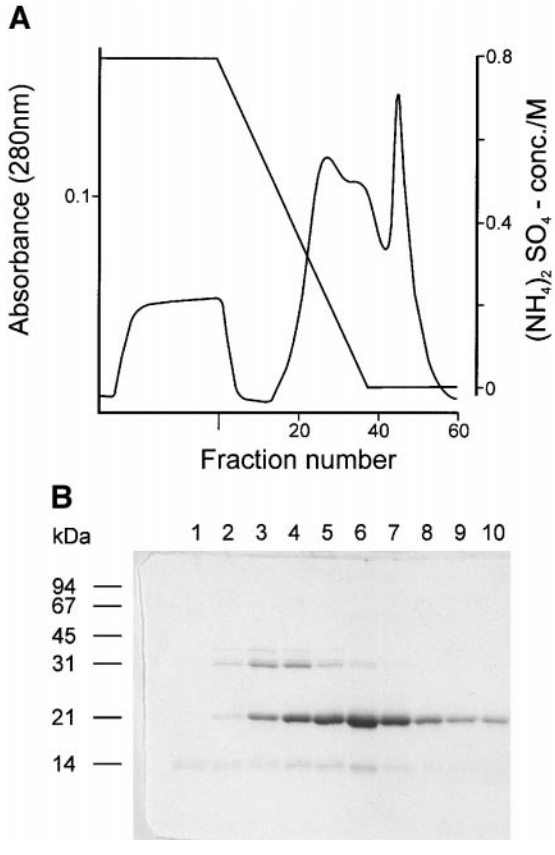

FIG. 2. (A) Phenyl-Sepharose chromatography of pool fractions 31–38 from DEAE Sepharose chromatography of HRBC hemolysate. Ammonium sulfate was added to pool fractions 31–38 from the DEAE ion exchange chromatography to a final concentration of 0.8 M. The sample was applied to the column and the column was washed with buffer A (0.8 M $(NH_4)_2SO_4$ in 20 mM Tris, pH 8.0). Elution was performed with a linear gradient from 0.8 to 0 M ammonium sulfate (buffer B, 20 mM Tris, pH 8.0). Pool fractions 39–45 and 46–54, the latter pool being the purer. (B) SDS–polyacrylamide gel electrophoresis in a 12% mini-slab gel of fractions from Phenyl Sepharose chromatography of pool fractions 31–38 from DEAE ion exchange chromatography. Lanes 1–10, fractions 30, 36, 40, 42, 44, 46, 48, 50, 52, and 54; MW standards are indicated.

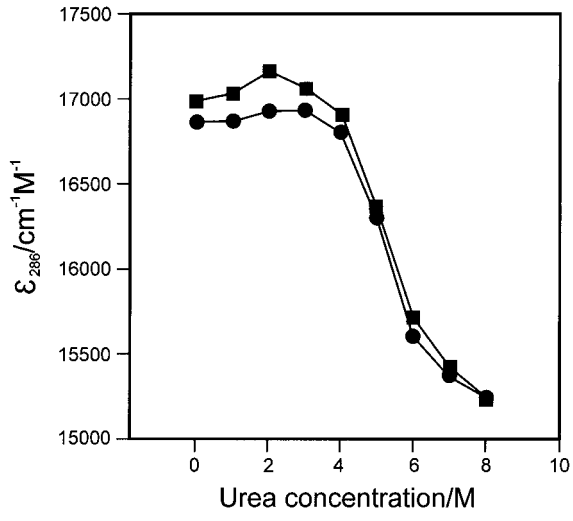

FIG. 3. Denaturation of calpromotin/TSA with urea in 25 mM potassium phosphate, pH 7.0, in the absence (squares) and in the presence (circles) of dithiothreitol.

0.03 thiols per mole of protein. After both denaturation and reduction we found 2.6 ± 0.09 thiols per molecule (averages of three preparations ± SD).

The function of the protein in the living cell is likely to be influenced by the dynamics of the interconversion between the two forms of the protein, the high molecular weight form and the dimeric form. As a first attempt to dissociate the multimer we decided to investigate the influence of urea at concentrations not leading to general denaturation. Figure 3 shows that no gross structural changes take place up to a concentration of urea of 3 M, and that the protein unfolds in the range between 3 and 7 M. It is noteworthy that reduction with dithiothreitol has no apparent effect on the unfolding curve.

Figure 4 shows HPLC gel-filtration experiments in the absence of urea and at four different urea concentrations, all in the absence of a reducing agent. The protein was incubated in the respective eluants prior to chromatography for about one hour. At 2.8 M urea the protein appeared exclusively as a species with a molecular size between 50 and 60 kDa. At 1 and 2 M urea it is observable that species of intermediate sizes are present. The experiments show directly that covalent bonds are not involved in multimer formation.

Gel-filtration experiments (Figs. 5 and 6) at pH 5.4 and 8.53 show that calpromotin is present as a large aggregate (about 300 kDa) at the lower pH value and as a species with a hydrodynamic radius equivalent to

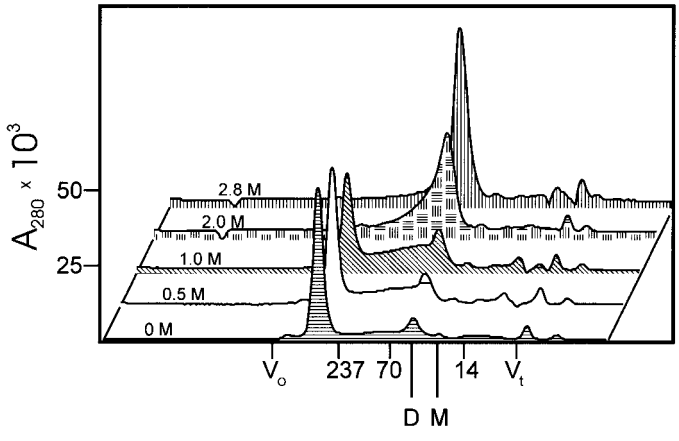

FIG. 4. Dissociation of the multimeric complex of calpromotin/TSA into dimers by urea studied by gel filtration. For each curve, the concentration of urea is given in the figure. V_0 is the void volume, and V_t is the position of acetone marker. Numbers at the abscissa refer to standard protein molecular weights, and D and M to expected positions of dimer and monomer, respectively.

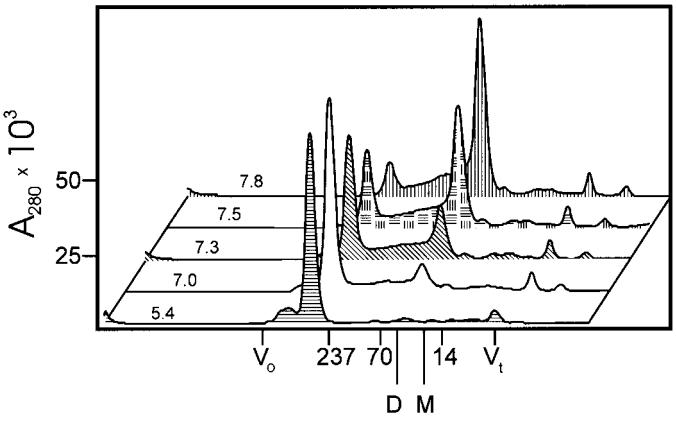

FIG. 5. Gel filtration of calpromotin/TSA at various pH values indicated in the figure. The amount of protein applied was 1.7 μg in each experiment. One experiment (at pH 8.5) included in Fig. 6 could not be inserted here, because it was obtained with another data collection system, but it showed one peak only at the position of dimers. V_0 is the void volume, and V_t is the position of acetone marker. Numbers at the abscissa refer to standard protein molecular weights, and D and M to expected positions of dimer and monomer, respectively

a molecular weight of about 50 kDa above pH 8.5. At intermediate pH values we found both species to be present in relative amounts which varied with the pH at which chromatography was carried out. This is not expected if the transition between the two forms is fast compared to the duration of the chromatographic experiment (1 h). Apparently, aggregation is governed by a dissociable group that titrates between pH 7 and 8.

DISCUSSION

The protein isolated in this study is most likely identical to the protein isolated earlier as calpromotin (2, 3, 6). It behaves identically during ion exchange chromatography, and it has the same molecular weight. The number of cysteines is also identical, and they show similar reactivities. In agreement with an earlier report (4) we find that the reactivity of the isolated and untreated protein toward DTNB is very small. After denaturation with SDS the presence of one thiol group per unit of 23 kDa was reported (4). Our value obtained after denaturation with urea is smaller (0.63 per 21.9 kDa). This could be due to the presence of impurities in the preparation or to a larger degree of oxidation or unknown derivatization of some of the cysteines during our preparation. After denaturation with SDS and reduction with DTT calpromotin most likely contains three cysteines (4). Our value is 2.6 cysteines per mole of protein, almost exactly 2 more thiols than after denaturation alone. This indicates that the reason for finding 0.63 thiol groups per peptide chain instead of one was due to some unknown derivatization and not to impurities. When the native protein was first reduced prior to thiol estimation, we found that it contained 1.7 thiol groups per mole of protein, very nearly one more than after denaturation alone.

If there are three cysteine residues per peptide chain in a dimer which is linked by disulfide bonds and one of the cysteines is in the reduced form, it follows logically that the other two cysteines must be involved in interchain disulfide bonds. Our measurements could then be explained in two ways. One is that reduction of the native protein affects only one of the disulfide bonds and that a concomitant change in structure exposes the buried, reduced cysteine residue. Another is that reduction of the native protein affects both disulfide bonds, but leaves the originally reduced cysteine in a nonexposed state. Only quantitative labeling experiments may answer this question.

The low reactivity of the native protein toward DTNB could be due to steric factors, because in the intact erythrocyte, calpromotin was found to be labeled with iodoacetate (2). Taking into account the high concentration of glutathione present in red cells, it cannot be excluded that one of the disulfide bridges present in the isolated protein is in fact reduced to thiol groups in the intact cell. These could then be the target for iodoacetate in the labeling experiments (2).This line of thinking is in agreement with the finding (11) that TSA protects against free radicals produced by the ascorbate/Fe(III) system only in the presence of DTT. Further, *N*-ethylmaleimide inactivates TSA only after reduction by DTT (11).

During gel filtration chromatography at pH 7.4 it was observed (4) that most of the calpromotin eluted with a retention time corresponding to a molecular size of about 300 kDa, while a minor fraction eluted with a

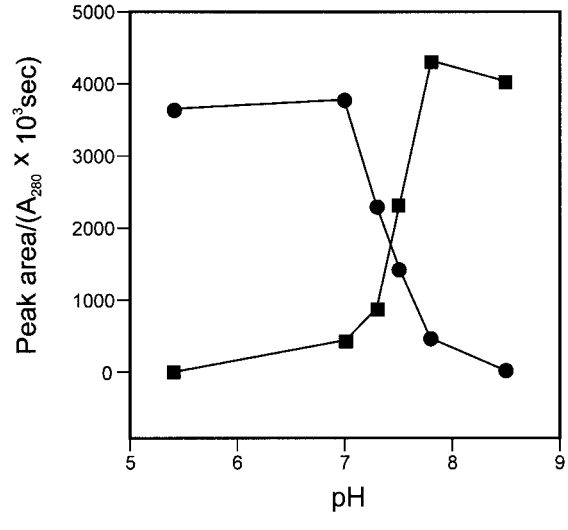

FIG. 6. The relative amounts of protein present as dimers (squares) or multimers (circles) as a function of pH, expressed as the areas under the curves of the respective peaks in the chromatograms of Fig. 5.

size of about 30 kDa. Also in our case calpromotin elutes with two different molecular weights. The larger form has a size not distinguishable from 300 kDa, while the smaller one in our case elutes with a retention time expected for a globular protein with a molecular weight of 45 kDa, indicating that the low molecular weight form in our case is a dimer (Fig. 5). On SDS–PAGE under nonreducing conditions we observe that our protein migrates as a dimer (data not shown) as is the case for calpromotin (4). We feel it safe to conclude that the protein isolated by us is in fact identical to the protein isolated as calpromotin (4).

The single tryptic peptide sequenced by us gave only one hit in a database search. In view of the obtained sequence (YVVLFFYPLDFTFV), it is highly unlikely that this sequence should be present in proteins that are not identical to or at least very related to thiol-specific antioxidant protein isolated from human brain (5). A homologous protein was originally isolated from yeast as a component that protects certain enzymes from oxidative damage by reactive oxygen species (10), and it was at that time named protector protein (PRP). More recently a protector protein was isolated from human red blood cells (11). The yeast protein was observed to exist in two forms with molecular weights of 500 and 90 kDa, respectively. PRP from human red blood cells is identical to the brain enzyme (12). The above comparisons lead to the conclusion that the protein isolated by us is identical both to calpromotin and to TSA or PRP.

The dissociation of the high molecular weight form by urea (Fig. 4) was performed in a range of urea concentrations that did not lead to denaturation of the protein as monitored by the blue-shift of the absorption spectrum. The experiments do not allow a determination of the number of subunits in the large complex, but they indicate that it is composed of dimers of the constituent peptide. The largest fraction of the dissociation of the complex occurs between 1 and 2 M urea, and it is quite evident that the association is due only to non-covalent forces, meaning that disulfide bridges are not involved. Formation of disulfide bridges are, however, most probably involved in the dimerization process, as evidenced from nonreductive SDS–PAGE.

The pH dependency of the equilibrium between dimers and the large complex (Fig. 6) indicates that a dissociable group with a p*K* value around 7.5 is of major importance. This does not necessarily mean that the complex is kept together by ionic forces directly. It is equally likely that the dissociation could be secondary to structural changes in the dimers induced by dissociation of the group in question. The gel-filtration experiments carried out at pH values intermediate between the fully associated and fully dissociated states indicate that attainment of equilibrium is a slow process. The relative rates of association and dissociation and the duration of gel filtration will all have significance for the exact interpretation of the gel-filtration experiments and the exact p*K* value obtained. In analogy with the kinetics of denaturation/renaturation, the interpretation is further complicated by the fact that the rate of approach to equilibrium will be dependent on how far the system is from equilibrium during attainment of equilibrium. But we consider it unlikely that the transition point should be outside the physiologically interesting range.

The results reported in this paper show that calpromotin belongs to a class of proteins that have central roles as radical scavengers in living cells, and that it is identical to one of these. In experiments aiming at reconstitution of membrane transport function it directs attention to the significance of the redox state in the system and to the care taken in avoiding oxidative damage.

It is also shown that the earlier observed existence of low- and high-molecular-weight forms of thiol-specific antioxidant proteins is due to a dynamic equilibrium between the two forms, which is governed by a dissociable group with a p*K* value in the physiological range. The quantitative description of the role of this equilibrium for the reactivity of the thiol groups involved in radical scavenging will be important in future experiments.

ACKNOWLEDGMENTS

We thank Ellen Storm Hansen and Gurli Bengtson for their expert technical assistance and Peter Jepsen for preparing the illustrations.

REFERENCES

1. Lauf, P. K., Bauer, J., Adragna, N. C., Fujise, H., Zade-Oppen, A. M., Ryu, K. H., and Dilpire, E. (1992) *Am. J. Physiol.* **263,** C917–C932.
2. Plishker, G. A. (1985) *Am. J. Physiol.* **248,** C419–C424.
3. Plishker, G. A., White, P. H., and Cadman, E. D. (1986) *Am. J. Physiol.* **251,** C535–C540.
4. Plishker, G. A., Chevalier, D., Seinsoth, L., and Moore, R. B. (1992) *J. Biol. Chem.* **267,** 21839–21843.
5. Lim, Y. S., Cha, M.-K., Kim, H.-K., and Kim I.-H. (1994) *Gene* **140,** 2790–2784.
6. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
7. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77.
8. Stone, K. L., LoPresti, M. B., Crawford, J. M., DeAngelis, R., and Williams, K. R. (1989) *in* A Practical Guide to Protein and Peptide Purification for Microsequencing (Matsudaira, P. T., Ed.), pp. 31–47, Academic Press, New York.
9. Moore, R. B., Plishker, G. A., and Shriver, S. K. (1990) *Biochem. Biophys. Res. Commun.* **166,** 146–153.
10. Kim, K., Kim, I.-H., Lee, K.-Y., Rhee, S. G., and Stadtman, E. R. (1988) *J. Biol. Chem.* **263,** 4704–4711.
11. Lim, Y. S., Cha, M.-K., Uhm, T. B., Park, J. W., Kim, K., and Kim, I.-H. (1993) *Biochem. Biophys. Res. Commun.* **192,** 273–280.
12. Cha, M.-K., and Kim, I.-H. (1995) *Biophys. Biochem. Res. Commun.* **217,** 900–907.