Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2236–2240, March 1991
Biochemistry

Identification and purification to near homogeneity of the vitamin K-dependent carboxylase

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Communicated by K. M. Brinkhous, December 26, 1990

ABSTRACT **Vitamin K-dependent carboxylase catalyzes the modification of specific glutamic acids to γ-carboxyglutamic acid in several blood-coagulation proteins. This modification is required for the blood-clotting activity of these proteins and has thus been the subject of intense investigation. We have now identified the bovine vitamin K-dependent carboxylase and purified it to near homogeneity by an affinity procedure that uses the 59-amino acid peptide FIXQ/S (residues −18 to 41 of factor IX with mutations Arg → Gln at residue −4 and Arg → Ser at residue −1). The carboxylase as purified has a molecular weight of 94,000. It is also the major protein that can be cross-linked to iodinated FIXQ/S and is the only protein whose cross-linking is prevented by a synthetic factor IX propeptide. The degree of purification is about 7000-fold with reference to ammonium sulfate-fractionated microsomal protein from liver.**

A number of blood coagulation proteins require a posttranslational vitamin K-dependent modification for biological activity. Stenflo *et al.* (1), Nelsestuen *et al.* (2), and Magnusson *et al.* (3) have reported that prothrombin, the prototype of these vitamin K-dependent proteins, contained the modified amino acid γ-carboxyglutamic acid (Gla). Prothrombin from animals treated with the vitamin K antagonist warfarin lacked this Gla modification. It was inferred from these observations that the blood-clotting activity of the vitamin K-dependent proteins required γ-carboxylation of specific glutamic acid residues. Shortly thereafter, Esmon *et al.* (4) demonstrated an enzyme activity, vitamin K-dependent carboxylase (hereafter called carboxylase), capable of making this Gla modification.

After cDNA sequences were obtained for several of the vitamin K-dependent proteins, Pan and Price (5) compared the deduced amino acid sequences and suggested that the propeptide consensus sequence preceding the amino terminus of the vitamin K-dependent protein was a recognition site for the carboxylase. This suggestion was confirmed by Knobloch and Suttie (6), who demonstrated the importance of the propeptide in carboxylation by showing that the synthetic propeptide sequence of human factor X stimulated the activity of the carboxylase for a small substrate (Boc-Glu-Glu-Leu-OMe) *in vitro*. Jorgensen *et al.* (7) extended this observation by showing that factor IX with its propeptide deleted was not carboxylated.

In spite of its importance, the carboxylase has not been previously purified. Purification of 400-fold was reported by Girardot (8). Comparison of Girardot's results to later purifications is complicated because, in our hands, ammonium sulfate and the propeptide stimulate the incorporation of CO_2 into the synthetic peptide substrate FLEEL by 13-fold. If one corrects for the lack of ammonium sulfate and propeptide in Girardot's assay mix, then he achieved a specific activity of 1.1×10^7 cpm per mg per hr. Soute *et al.* (9) demonstrated that an immobilized factor X antibody would bind the carboxylase, presumably through a factor X precursor–carboxylase complex, and that the bound carboxylase retained its activity for the synthetic peptide substrate FLEEL. Harbeck *et al.* (10) extended this method by eluting the carboxylase from a prothrombin antibody column with a synthetic propeptide achieving a 500-fold purification and a final specific activity of 6.6×10^6 cpm per mg per hr. Hubbard *et al.* (11) reported the purification of the carboxylase to homogeneity using a synthetic propeptide sequence as an affinity ligand. However, the reported final specific activity of 1.3×10^7 cpm per mg per hr was still not significantly different than that reported by Girardot (8). Numerous studies with the crude carboxylase have yielded important information about its properties and mode of action (12). It is clear, however, that for detailed mechanistic studies and physical characterization of the enzyme, purification is necessary.

We recently reported the production in *Escherichia coli* of four 59-residue peptides containing the propeptide and Gla domain of human factor IX (13). We report here that one of these peptides, FIXQ/S (residues −18 to 41 of factor IX with mutations Arg → Glu at residue −4 and Arg → Ser at residue −1), is an excellent affinity ligand for purification of the carboxylase. A 7000-fold purification of the carboxylase to 80–90% apparent purity and final specific activity of about 2.4×10^9 cpm per mg per hr was obtained. The apparent molecular weight was 94,000 by reducing SDS/PAGE analysis.

MATERIALS AND METHODS

All chemicals are reagent grade. 3,3'-Dithiobis(sulfosuccinimidylpropionate) was purchased from Pierce. Aprotinin and pepstatin A were purchased from Boehringer Mannheim. Leupeptin, phenylmethylsulfonyl fluoride, and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) were obtained from Sigma. The peptide FLEEL and the protease inhibitors FFRCK and FPRCK were from Bachem. Peptide proFIX19, AVFLDHENANKILNRPKRY, was synthesized by Frank Church (University of North Carolina at Chapel Hill). $NaH^{14}CO_3$, specific activity 50.0 mCi/mmol (1 Ci = 37 GBq), was from NEN and Aqua Mephyton was from Merck Sharp & Dohme. Protease inhibitor mixture (PIC) was freshly prepared as a 10× PIC stock containing 20 mM dithiothreitol, 20 mM EDTA, FFRCK (1.25 μg/ml), FPRCK (1.25 μg/ml), leupeptin (5 μg/ml), pepstatin A (7 μg/ml), phenylmethylsulfonyl fluoride (340 μg/ml), and aprotinin (20 μg/ml).

Preparation of Affinity Column. Peptide FIXQ/S (13) with two mutations in the propeptide was chosen for the affinity

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Abbreviations: FIXQ/S, residues −18 to 41 of factor IX with mutations Arg → Gln at residue −4 and Arg → Ser at residue −1; Gla, γ-carboxyglutamic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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ligand because its affinity for the carboxylase is not changed and because it has fewer trypsin cleavage sites than our other peptides and is, therefore, less likely to be degraded by proteases in the crude extracts used for purification. Peptide FIXQ/S was prepared according to Wu *et al.* (13). FIXQ/S (100 mg) was coupled to 25 ml of Affi-Gel 10 (Bio-Rad) according to the manufacturer. The reaction was done at pH 4.8, which is one unit below the theoretical pI of FIXQ/S. The final concentration of the covalently bound FIXQ/S on Affi-Gel 10 was measured as 442 μM and the coupled ligand is referred to as Affi-FIXQ/S.

Affinity Purification of Carboxylase. Preparation of microsomes from bovine liver, solubilization of microsomes, and ammonium sulfate fractionation were as described by Girardot (8). Protein concentration was measured with the Bio-Rad protein assay kit (14). Alternatively, protein concentration and relative purity were determined by scanning of SDS/PAGE gels (15) stained with silver (16) or Coomassie blue. In all cases, reference curves were prepared with IgG as a standard. Ammonium sulfate-fractionated microsomal protein (7–8 g) was resuspended in buffer A [25 mM Mops, pH 7.0/500 mM NaCl/20% (vol/vol) glycerol/0.1% phosphatidylcholine/1× PIC]/0.1% CHAPS to a total volume of 150 ml. In all purification experiments, except that depicted in Fig. 1, lane 3, the suspension was sonicated with a standard ultrasonic probe (sonicator model W-220F, Heat Systems/Ultrasonics) at scale 9 for 100 2-sec pulses in an ice bath. The sonicated material was loaded on a 25-ml Affi-FIXQ/S column equilibrated with 150 ml of 0.1% CHAPS/buffer A at 4°C at a flow rate of 10 ml/hr. The loaded column was washed with 100–200 ml of 0.1% CHAPS/buffer A. The carboxylase was eluted from the Affi-FIXQ/S column by one of the following methods.

Elution I. Propeptide (150 μM) in buffer A/0.1% CHAPS was used to elute the carboxylase. The column was filled with eluant and incubated overnight before collecting the eluate. The propeptide eluate was concentrated with Centricon-30 (Amicon); the filtrate could be reused for elution. Significant amounts of carboxylase activity were continuously eluted for a week.

Elution II. A flow rate of 10 ml/hr was used for all chromatography steps. An extensive wash was carried out by two sequential steps: a 100-ml 0.05–0.85% Triton X-100 gradient in buffer B (25 mM Mops, pH 7.0/50 mM NaCl/20% glycerol/1× PIC) containing 0.2% phosphatidylcholine was followed by a 100-ml 0.1–1% CHAPS gradient in buffer B containing 0.1% phosphatidylcholine. Elution was accomplished by using 100 ml of a double gradient containing 0.1–1% CHAPS and 0–2 μM proFIX19 in buffer A and continued with another 100 ml containing 1% CHAPS and 2 μM proFIX19 in buffer A.

CM-Sepharose Chromatography. Desalted concentrated carboxylase (total activity of 2.2×10^7 cpm/30 min) eluted by method I was prepared in buffer C (25 mM Mops, pH 7.0/100 mM NaCl/20% glycerol/1× PIC) containing 1% Triton X-100 and 0.7% phosphatidylcholine to a total volume of 3.85 ml. This carboxylase preparation was then sonicated in a bath sonicator for twenty-four 5-sec pulses and batch-adsorbed to 5.8 ml of CM-Sepharose. The CM-Sepharose was packed into a column, washed, and eluted with a 10-ml 100–450 mM NaCl gradient in buffer C containing 0.05% Triton X-100 and 0.2% phosphatidylcholine.

Cross-Linking Reaction. The cross-linking was accomplished with 3,3′-dithiobis(sulfosuccinimidyl propionate) as described by Jung and Moroi (17). For competition experiments, 100-fold excess of proFIX19 was included.

Carboxylase Assay. The assay was done for 30 min as described (13) in 25 mM Mops, pH 7.0/500 mM NaCl/0.16% CHAPS/0.16% phosphatidylcholine. FLEEL at 3.6 mM and 5 μCi of $NaH^{14}CO_3$ were the substrates; 0.8 M ammonium sulfate and 16 μM proFIX19 were included as activators.

RESULTS

Identification of the Carboxylase. Fig. 1 displays a reducing SDS/PAGE analysis of our initial Affi-FIXQ/S purification of carboxylase. There is little difference between the protein pattern of the starting material and the flow-through (lanes 1 and 2, respectively). The sample in lane 3 represents protein eluted with 150 μM proFIX19. There is some enhancement of a protein band at M_r 94,000 but the purification achieved is only 60-fold. We reasoned that the poor purification might be the result of large micelles that contained at least one carboxylase molecule as well as other integral membrane proteins. The carboxylase molecule would allow binding to the affinity matrix but the other proteins would be coeluted with the carboxylase. We tried to apply the standard solution to this problem, which is to increase the detergent concentration until one protein per micelle is achieved. However, the carboxylase would not bind to Affi-FIXQ/S in the presence of a high concentration of a number of detergents. Therefore, we sought to reduce the micelle size by sonicating the starting material. As shown in Fig. 1, lane 4, the relative intensity of the M_r 94,000 band in the proFIX19 eluate is much more prominent after sonication. Furthermore, the amount of carboxylase bound to Affi-FIXQ/S increased from 15 to 30% and the purification increased to 500-fold.

To evaluate the binding specificity of FIXQ/S and attain an independent estimate of the molecular weight of the carboxylase, we cross-linked ^{125}I-labeled FIXQ/S to 60-fold affinity-purified material (Fig. 1, lane 3). The autoradiogram in Fig. 2, lane 2, shows one major band, slightly larger than M_r 97,000 that represents the complex of the cross-linked protein and ^{125}I-labeled FIXQ/S. This major band was eliminated when the same experiment was run in the presence of excess nonradioactive proFIX19 (Fig. 2, lane 1). The minor bands in lane 2 were unaffected by competition (lane 1). By subtracting the molecular weight of FIXQ/S (M_r 7000) from the estimated size of the cross-linked complex, we estimate that the size of the protein to which the FIXQ/S peptide binds is approximately M_r 94,000. This agrees with the size of the band enriched in each of the early purifications (Fig. 1), indicating that the M_r 94,000 protein (lanes 3 and 4) is the enzyme carboxylase and that the interaction between the peptide FIXQ/S and carboxylase is very specific.

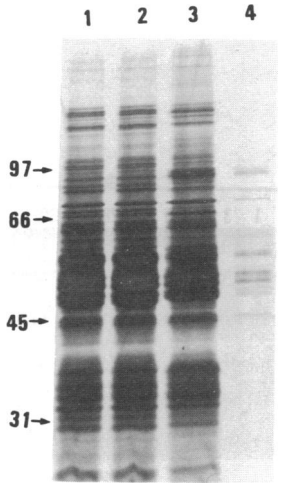

FIG. 1. Reducing SDS/PAGE analysis (silver-stained 10% gel) of Affi-FIXQ/S-purified carboxylase preparations demonstrate the effect of sonication. The cpm reflect the carboxylase activity in each lane. Lane 1 is the sonicated loading material (380 cpm/30 min); lane 2 is the sonicated flow-through (265 cpm/30 min); lane 3 is unsonicated elution I carboxylase preparation (23,500 cpm/30 min); lane 4 is sonicated elution I carboxylase preparation (5000 cpm/30 min). Molecular weights ($\times 10^{-3}$) are shown to the left.

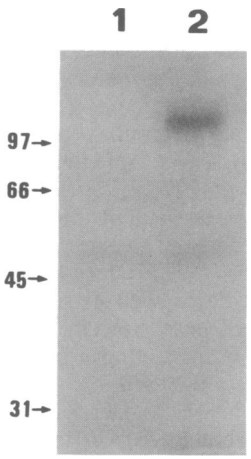

FIG. 2. Autoradiogram of cross-linking between ^{125}I-labeled FIXQ/S and protein from the partially purified carboxylase preparation shown in Fig. 1, lane 3. Proteins were separated by nonreducing SDS/PAGE on a 10% gel. Lane 1 is proFIX19 competition of cross-linking; lane 2 is cross-linking in absence of proFIX19. Molecular weights ($\times 10^{-3}$) are shown to the left.

Results shown in Fig. 3 indicate that further purification can be achieved by chromatography of the 500-fold affinity-purified material on CM-Sepharose. Resolubilization, sonication, and batch adsorption on CM-Sepharose followed by elution with a salt gradient produced a dramatic improvement in purification. The batch adsorption step is very important as enzymatic activity is lost and significantly less purification is achieved when the material is adsorbed to the top of the column. However, the most important information in this figure is that the carboxylase activity in each fraction is proportional to the amount of M_r 94,000 protein in the corresponding SDS/PAGE analysis. Quantitation of the silver-stained gel by scanning reveals that the protein is 80–90% pure. The specific activity is 2×10^9 cpm per mg per hr.

Further evidence that the M_r 94,000 band represents the carboxylase is presented in Fig. 4. A carboxylase preparation purified by Affi-FIXQ/S and CM-Sepharose (lane 2) was reapplied to the Affi-FIXQ/S column. Lane 3 shows that the activity of carboxylase was removed when the M_r 94,000 protein bound to Affi-FIXQ/S. Lane 4 shows that the activity again coeluted with the M_r 94,000 protein. This carboxylase is from one of our earlier preparations and contained a significant amount of inactive carboxylase. This explains the observation that the amounts of protein in the M_r 94,000 bands in lanes 2 and 4 are approximately equal whereas the activity is greater in the sample repurified by Affi-FIXQ/S; only active carboxylase binds to the affinity column.

Purification of the Carboxylase. One of the problems with using the affinity column is that the elution using the propeptide is very slow and requires several days to achieve an adequate yield. We therefore explored alternative methods to elute the enzyme from the affinity column. As shown in Fig. 5, we were able to remove essentially all contaminating proteins without loss of carboxylase activity by washing the loaded affinity column extensively with Triton X-100 followed by a wash with CHAPS. The carboxylase could then be eluted with a gradient of CHAPS in a high concentration of NaCl (data not shown) or a gradient of CHAPS and propeptide containing a high concentration of NaCl (Fig. 5). The carboxylase activity coincides with the M_r 94,000 protein profile. And, depending upon the fraction chosen, the carboxylase is 80–95% pure. If the propeptide is omitted from the elution gradient, the carboxylase in higher concentrations of CHAPS rapidly loses activity. Table 1 shows our final purification scheme. Note that 30% of the activity in the starting material is bound (obtained by subtracting the activity in the flow-through from the load) and that 34% of total activity is recovered by elution. Thus it appears that very little of the observed increase in specific activity is due to the removal of an inhibitor.

DISCUSSION

We have affinity-purified the carboxylase to 80–90% purity with a yield of 34%. The procedure is rapid and efficient. The entire purification can be accomplished in 4 days. It is difficult to compare different purification schemes but, by comparing final specific activities, it appears that our puri-

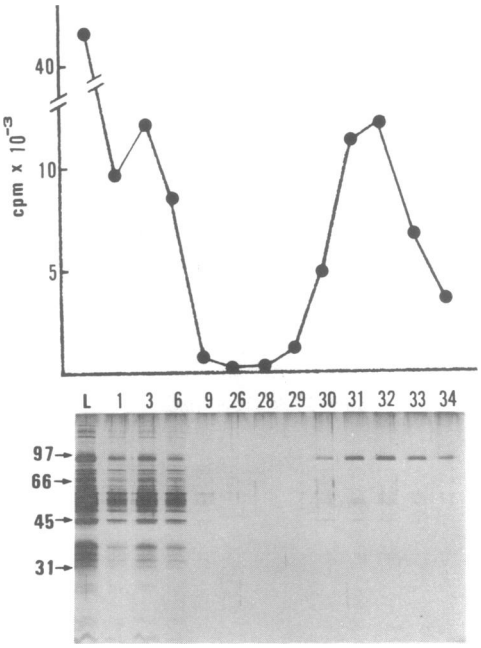

FIG. 3. Activity profile (*Upper*) and reducing SDS/PAGE analysis (10% silver-stained gel) of fractions from CM-Sepharose chromatography (*Lower*). The fraction numbers are on the *x* axis. A 7.5-μl sample from each fraction was analyzed by SDS/PAGE. The cpm, shown on the *y* axis, represent the carboxylase activity in each lane. The carboxylase activity was determined by the $^{14}CO_2$ incorporation into FLEEL in the standard assay. Lane L is loading material; fractions 1–7 are flow-through; fractions 8–27 are wash; fractions 28–37 are elution. Molecular weights ($\times 10^{-3}$) are shown to the left.

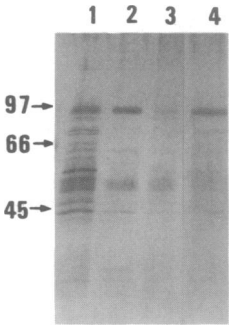

FIG. 4. Reducing SDS/PAGE analysis (silver-stained 10% gel) of a CM-Sepharose-purified carboxylase rechromatographed on Affi-FIXQ/S to show the correlation between the M_r 94,000 protein and activity. Lane 1 is the first Affi-FIXQ/S carboxylase preparation (9750 cpm/30 min); lane 2 is purified CM-Sepharose eluate (6729 cpm/30 min, used for the second Affi-FIXQ/S); lane 3 is flow-through of the second Affi-FIXQ/S (394 cpm/30 min); lane 4 is sample of second Affi-FIXQ/S elute (18853 cpm/30 min, eluate I method). The difference in activity between the carboxylase shown in lanes 2 and 4 is the result of some inactivation of the carboxylase of lane 2. Molecular weights ($\times 10^{-3}$) are shown to the left.

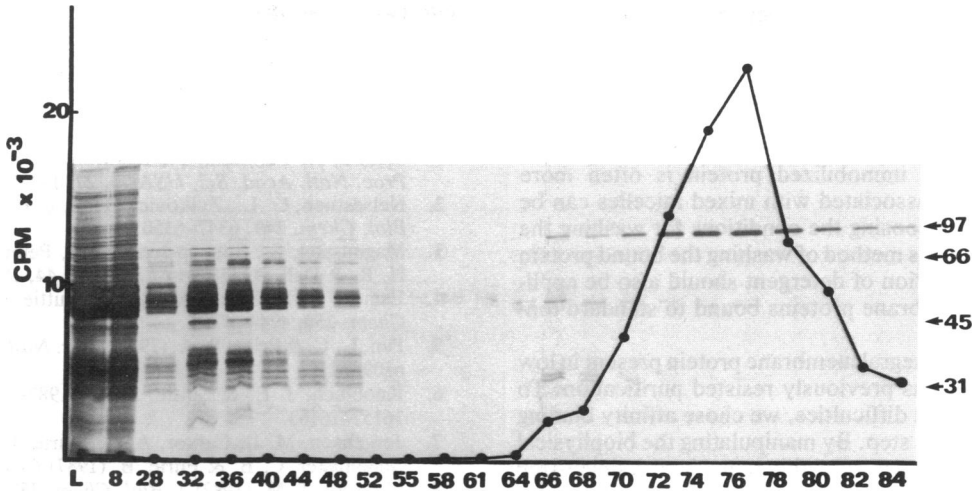

FIG. 5. Activity profile and reducing SDS/PAGE analysis (10% silver-stained gel) of fractions from Affi-FIXQ/S chromatography elution II. A 5.0-μl sample of each fraction was used for SDS/PAGE analysis except that the loading material and fraction 8 were diluted 1:100 before analysis. The fraction number is shown on the x axis. The y axis represents the carboxylase activity in each lane. The carboxylase activity was determined by the $^{14}CO_2$ incorporation into FLEEL in the standard assay. Lane L is loading material; fractions 1–20 are flow-through (because each fraction is equivalent, only one is shown); fractions 21–30 are wash; fractions 31–49 are Triton X-100 gradient; fractions 56–63 are CHAPS gradient; fractions 64–75 are CHAPS/proFIX19 double gradient; fractions 76–90 are 1% CHAPS/2 μM proFIX19 elution. Molecular weights ($\times 10^{-3}$) are shown on the right.

fication results in a carboxylase preparation that 185-fold higher specific activity than the best previously published method (11). From $^{14}CO_2$ incorporation into the pentapeptide FLEEL, we estimate the specific activity of our carboxylase prepared by Affi-FIXQ/S chromatography eluted by method II (Fig. 5) as 2.4×10^9 cpm per mg of protein per hr. This represents a 7000-fold purification. Hubbard *et al.* (11) reported a specific activity of 1.3×10^7 cpm per mg of protein per hr with a 107-fold purification from their affinity purification step. The results should be comparable, because the CO_2 used in the two experiments had the same specific activities.

When the purified carboxylase is analyzed by nonreducing SDS/PAGE, there is no evidence of a higher molecular weight disulfide-bonded multimer. In nonreducing gels, however, there is a tight doublet of about M_r 94,000 that we believe are conformers of the carboxylase. This suggestion is supported by experiments (D.P.M., unpublished data) in which limited tryptic digests of the M_r 94,000 protein yielded only two polypeptides of approximately M_r 30,000 and M_r 64,000. This observation provides additional evidence that there is only one M_r 94,000 protein in our preparation. It has been reported that the carboxylase is inhibited by the sulfhydryl reagent *p*-hydroxymercuribenzoate (12), and Price (18) has suggested that the carboxylase makes a disulfide bond with its normal substrate. Thus alternative reduced forms of the enzyme are not unexpected.

The major protein that coincides with the carboxylase activity in our preparation has a M_r 94,000 in reducing SDS/PAGE analysis. This is very different from the M_r 77,000 protein reported by Hubbard *et al.* (11). Flynn *et al.* (19) demonstrated that many different small peptides could be used for the single-step affinity purification of BiP (or glucose-regulated protein, or hsp78) from solubilized microsomes. Hubbard *et al.* (11), using a similar starting material and a similar purification scheme, obtained a protein with the same molecular weight as BiP with a relatively low specific activity for carboxylase. We concluded that the M_r 77,000 protein reported by Hubbard *et al.* (11) was BiP, one of the most abundant proteins in the endoplasmic reticulum. We tested BiP (obtained from Greg Flynn, Princeton University) for *in vitro* γ-carboxylation and conclude that it does not have carboxylase activity (data not shown). We have observed no significant binding of BiP to Affi-FIXQ/S. There is also no evidence of a protein at M_r 77,000 in the elution fractions 70–82 of Fig. 5 and FIXQ/S was not cross-linked to a protein of this size. Furthermore, a monoclonal anti-BiP antibody (obtained from David Bole, University of Michigan) failed to detect BiP on a Western blot of our propeptide-eluted carboxylase (60-fold purified; data not shown). Because of the amount of material loaded, we probably would not have detected BiP if it were present at 1–2%. It is likely that BiP tends to bind small peptides or that the peptide used by Hubbard *et al.* (11) happened to have a strong affinity for BiP. In addition to the M_r 78,000 glucose-regulated protein BiP, there is a M_r 94,000 glucose-regulated protein (20) that is present in relatively high concentrations and is, therefore, a candidate for a contaminant in our preparation. However, the amino acid sequence of five tryptic peptides of the carboxylase clearly demonstrates that we have not purified the M_r 94,000 glucose-regulated protein or, in fact, any other known protein.

The method used in this paper for purification of the carboxylase may have general importance for the purification

Table 1. Purification of carboxylase

Sample	Total protein, mg	Total carboxylase activity, cpm/30 min	Recovery of activity, %	Specific activity, cpm per mg per hr	Fold purification
Solubilized microsomes (load)	8100	1.14×10^9	100	2.81×10^5	1
Flow-through of Affi-FIXQ/S	8090	8.08×10^8	70	2×10^5	0.7
Bound to Affi-FIXQ/S	4.7	3.3×10^8	30	1.4×10^8	502
Affinity-purified carboxylase	0.402	3.88×10^8	34	1.93×10^9	7000

of membrane proteins. A basic strategy for purifying integral membrane proteins is to make micelles containing only one protein. However, high concentrations of detergent often result in the inactivation of the protein of interest. By appropriate sonication, one can create smaller mixed micelles without affecting the catalytic or binding ability of the protein. Because the immobilized protein is often more stable, the problems associated with mixed micelles can be solved by carefully choosing the conditions for washing the bound solid phase. This method of washing the bound protein with a high concentration of detergent should also be applicable to integral membrane proteins bound to standard ion-exchange matrices.

Carboxylase is an integral membrane protein present in low concentrations that has previously resisted purification. To overcome the inherent difficulties, we chose affinity binding as the first purification step. By manipulating the biophysical properties of micelles, we were able to achieve a single-step purification of carboxylase with 80–90% purity. Starting from 8 g of microsomal protein, we can easily generate 300–400 μg of carboxylase from a 25-ml Affi-FIXQ/S column, with a final yield of 34% of the starting activity. Glycerol, which is often used to stabilize the membrane protein during purification, proved to be an important stabilizer for carboxylase. It increases the thermal stability of carboxylase and also increases the half-life of carboxylase at 4°C. Although 20% glycerol does not inhibit carboxylase binding to Affi-FIXQ/S, it does prevent carboxylase from being eluted by proFIX19 and also inhibits the enzyme activity. The purified carboxylase is very stable and can be stored at −70°C for months without loss of activity. This work provides the necessary basis for genetic and mechanistic studies of this important enzyme.

We thank Robert Bell (Duke University) and Harold Roberts (University of North Carolina at Chapel Hill) for helpful discussions, David Bole (University of Michigan) for a monoclonal antibody to BiP, Greg Flynn (Princeton University) for a gift of BiP, Frank Church (University of North Carolina at Chapel Hill) for synthesizing peptides, and Dan Frazier for preparing large quantities of microsomes. This work was supported by Grant HL06350-29 from the National Institutes of Health.

1. Stenflo, J., Fernlund, P., Egan, W. & Roepstorff, P. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2730–2733.
2. Nelsestuen, G. L., Zytkovicz, T. H. & Howad, J. B. (1974) *J. Biol. Chem.* **249**, 6347–6350.
3. Magnusson, S., Sottrup-Jensen, L., Petersen, T. E., Morris, H. R. & Dell, A. (1974) *FEBS Lett.* **44**, 189–193.
4. Esmon, C. T., Sadowski, J. A. & Suttie, J. W. (1975) *J. Biol. Chem.* **250**, 4744–4748.
5. Pan, L. C. & Price, P. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6109–6113.
6. Knobloch, J. E. & Suttie, J. W. (1987) *J. Biol. Chem.* **262**, 16157–16163.
7. Jorgensen, M. J., Cantor, A. B., Furie, B. C., Brown, C. L., Shoemaker, C. B. & Furie, B. (1987) *Cell* **48**, 185–191.
8. Girardot, J.-M. (1982) *J. Biol. Chem.* **257**, 15008–15011.
9. Soute, B. A. M., Vermeer, C., de Metz, M., Hemker, H. C. & Kijnen, H. R. (1981) *Biochim. Biophys. Acta* **676**, 101–107.
10. Harbeck, M. C., Cheung, A. Y. & Suttie, J. W. (1989) *Thromb. Res.* **56**, 317–323.
11. Hubbard, B. R., Ulrich, M. M. W., Walsh, C., Furie, B. & Furie, B. C. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6893–6897.
12. Suttie, J. W. (1985) *Annu. Rev. Biochem.* **54**, 459–477.
13. Wu, S.-M., Soute, B., Vermeer, C. & Stafford, D. W. (1990) *J. Biol. Chem.* **265**, 13124–13129.
14. Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254.
15. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
16. Blum, H., Beier, H. & Gross, H. J. (1987) *Electrophoresis* **8**, 93–99.
17. Jung, S. M. & Moroi, M. (1983) *Biochim. Biophys. Acta* **761**, 152–162.
18. Price, P. A. (1988) *Annu. Rev. Nutr.* **8**, 565–583.
19. Flynn, G. C., Chappell, T. G. & Rothman, J. E. (1989) *Science* **245**, 385–390.
20. Mazzarella, R. A. & Green, M. (1987) *J. Biol. Chem.* **262**, 8875–8883.