quired for either $Zn^{2+}$ or $Cd^{2+}$. In the production of red light, the bulk of the luciferase active site is probably still intact, and the spectral shift is more likely to be caused by the change in the mode of luciferin binding to luciferase. Among the several groups in the protein which could interact with the heavy metal ions, sulfhydryl groups seem to be the most reactive, especially toward $Hg^{2+}$ (Gurd and Wilcox, 1956). Thus the sulfhydryl group would appear to play an important role in the binding of luciferin for the production of the typical yellow-green light.

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# Chromatography and Activity of Thiol-Subtilisin*

Laszlo Polgar and Myron L. Bender

ABSTRACT: Thiol-subtilisin, in which a serine side chain at the active site was replaced by a cysteine residue, was prepared from chromatographically purified subtilisin. The thiol derivative was separated from native subtilisin by ion-exchange chromatography. The unexpectedly marked difference in chromatographic behavior indicates an alteration in some of the physical properties of the protein molecule. Pure thiol-subtilisin exhibits a very low activity toward *p*-nitrophenyl *N*-benzyloxycarbonylglycinate which is a specific substrate for subtilisin. This low activity is interpreted in terms of a distortion of the active site of the thiol-enzyme rather than in terms of a difference in chemical reactivity between hydroxyl and sulfhydryl groups.

In previous papers (Polgar and Bender, 1966, 1967; Neet and Koshland, 1966) it was shown that the serine residue at the active site of subtilisin can be transformed into a cysteine side chain. This thiol derivative of subtilisin retains enzymatic activity when tested with substrates possessing a good leaving group such as nitrophenyl esters or *N-trans*-cinnamoylimidazole (Polgar and Bender, 1967). Quantitative values are only available for the hydrolysis of *p*-nitrophenyl acetate and *N-trans*-cinnamoylimidazole (Polgar and Bender, 1967) since some extraneous enzymatic activity has interfered with other substrates. The present paper was aimed at obtaining thiol-subtilisin of a high purity in order to study the hydrolysis of *p*-nitrophenyl *N*-benzyloxycarbonylglycinate, a more specific substrate.

* From the Institute of Biochemistry of the Hungarian Academy of Sciences, Budapest, Hungary, and from the Department of Chemistry, Northwestern University, Evanston, Illinois. *Received August 30, 1968.* Support of this work by the Hungarian Academy of Sciences and by the National Institutes of Health is gratefully acknowledged.

## Experimental Section

Subtilisin, Bacterial Proteinase Novo, was a produc

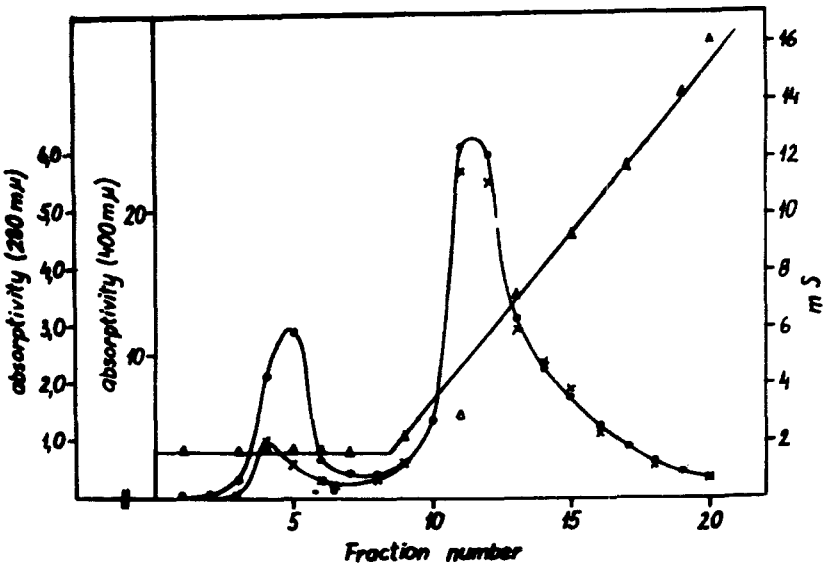


FIGURE 1: Chromatography of Novo subtilisin on carboxymethylcellulose at pH 7.0 using linear gradient elution with phosphate buffers. (○) Protein concentration at $E_{280}$; (+) enzymatic activity at $E_{400}$; (△) conductivity, m$S$.

of the Novo Pharmaceutical Co., Ltd. *p*-Nitrophenyl acetate was synthesized from *p*-nitrophenol and acetic anhydride in pyridine. It was recrystallized from alcohol–water and melted at 77.5–78.0°. *p*-Nitrophenyl *N*-benzyloxycarbonylglycinate was purchased from Cyclo Chemical Co.; phenylmethanesulfonyl fluoride from Calbiochem, Inc.; *p*-mercuribenzoate from the British Drug Houses Ltd.; thiolacetic acid and *N*-ethylmaleimide from Dr. Theodor Schuchardt GMBH et Co. Whatman CM11 carboxymethylcellulose was used for ion-exchange chromatography.

The concentration of subtilisin was measured spectrophotometrically using the data of Matsubara *et al.* (1965): $A^{1\%}_{278}$ 11.7, mol wt 27,600. The same data were employed with thiol-subtilisin.

The enzyme preparations were chromatographed on a carboxymethylcellulose column (0.9 × 15 cm) equilibrated with appropriate buffer. The ionic strength and the pH of the protein solutions were always adjusted by gel filtration prior to application to the ion-exchange column. The ionic strength and the pH of all solutions used in chromatography were checked with a conductivity meter (Radelkis, Type 932) and with a pH meter (Radelkis, Type OP-203), respectively. After the application of the enzyme in 0.01 M phosphate buffer to the column, the column was washed with the same buffer. This was followed by a linear gradient elution in which 20 ml of 0.1 M phosphate buffer was mixed to 20 ml of 0.01 M phosphate buffer. Fractions (2.5–3.0 ml) were collected.

The activity of subtilisin and thiol-subtilisin toward *p*-nitrophenyl esters was determined spectrophotometrically by measuring the liberation of *p*-nitrophenolate ion at 400 mμ in 0.1 M phosphate buffer (pH 7.5). Per cent activity due to thiol-subtilisin was determined from activity measurements in the absence and in the presence of thiol reagent. Thiol-subtilisin was inactivated by preincubation with 2–10 equiv of *N*-ethylmaleimide or *p*-mercuribenzoate prior to the addition of the substrate. Preincubation with *N*-ethylmaleimide took 2–3 min; *p*-mercuribenzoate reacted instantaneously.

## Results

*Chromatography of Subtilisin.* As the first step in preparing pure thiol-subtilisin the original subtilisin (Novo) preparation was analyzed. Figure 1 shows the chromatography of commercially available crystalline bacterial protease on a carboxymethylcellulose column. Phosphate buffers (0.01 and 0.1 M) (pH 7.0) were employed for linear gradient elution. Seven per cent of the total enzymatic activity as assayed with *p*-nitrophenyl *N*-benzyloxycarbonylglycinate as the substrate and 18% of the total absorbance at 280 mμ were recovered in the first peak. The second peak eluted by the gradient contained the main protein fraction with a higher specific activity and appeared to be homogeneous.

Subtilisin, not completely bound under the above conditions, and/or some other esterase may account for the hydrolytic activity of the first peak in Figure 1. To distinguish between these possibilities, the pooled fractions of the first peak were rechromatographed under the same conditions. No significant binding was observed. This implies that the bacterial protease preparation contains some hydrolytic enzyme activity which is different from subtilisin.

In accordance with the above idea, it was found that, during rechromatography of the second peak, 99% of the total enzymatic activity was bound to carboxymethylcellulose and could only be eluted at a higher buffer concentration. While only 1% of the activity of the chromatographically homogeneous subtilisin was not bound, 8–10% of the total absorption at 280 mμ passed through the column. This suggests that during pretreatment to rechromatography, *i.e.*, gel

TABLE I: Activity of Subtilisin and Thiol-subtilisin toward *p*-Nitrophenyl *N*-Benzyloxycarbonylglycinate in the Presence of Sulfhydryl Reagents.[a]

| | $\Delta E_{400}$/min | % Inhibn |
|---|---|---|
| Subtilisin | 45,000[b] | |
| + *p*-Mercuribenzoate | 45,000[b] | |
| + *N*-Ethylmaleimide | 45,000[b] | |
| Thiol-subtilisin preparation | 196 | |
| + *p*-Mercuribenzoate | 90 | 54 |
| + *N*-Ethylmaleimide | 96 | 51 |
| Thiol-subtilisin purified by chromatography | 175 | |
| + *p*-Mercuribenzoate | 68 | 61 |
| + *N*-Ethylmaleimide | 70 | 60 |

[a] Enzyme ($5 \times 10^{-6}$ M), *p*-nitrophenyl *N*-benzyloxycarbonylglycinate ($1 \times 10^{-4}$ M), *p*-mercuribenzoate or *N*-ethylmaleimide ($5 \times 10^{-5}$ M), and acetonitrile (3.3%) in 0.1 M phosphate buffer (pH 7.5). [b] Calculated value for $5 \times 10^{-6}$ M subtilisin concentration.

filtration and lyophilization, some of the subtilisin is denaturated. Accordingly, a second rechromatography of the subtilisin fraction yields the same pattern as in the first rechromatography.

The recovery of subtilisin based on the dry weight of the commercial preparation is about 75% as determined from the absorption of the enzyme solution at 280 mμ. The yield decreased to 50% after gel filtration, and about 40% can be eluted from the carboxymethylcellulose column as homogeneous subtilisin.

If we carry out the purification procedure at pH 6.5 rather than at pH 7.0, a similar chromatogram is obtained except that subtilisin not bound to the carboxymethylcellulose column is somewhat less than 1% in the rechromatography and also that denaturation appears to be lower than was found at the higher pH value. Even though the conditions are apparently more favorable at pH 6.5, the purification of subtilisin was routinely carried out at pH 7.0 in order to make sure that the extraneous hydrolase activity was removed.

The higher stability of subtilisin at pH 6.5 where its hydrolytic activity is lower suggests that denaturation may be due to autodigestion. This idea is supported by the long tailing of the protein into the low molecular weight fraction upon gel filtration. If this is indeed the case, the purification of an inactive derivative of subtilisin should be more successful than that of the native enzyme. In fact, Matsubara *et al.* (1965) crystallized diisopropylphosphoryl-subtilisin to determine the physical properties and the amino acid composition of subtilisin. Luckily, thiol-subtilisin is prepared from a similar inactive derivative, phenylmethylsulfonyl-subtilisin (Polgar and Bender, 1966; Neet and Koshland, 1966). Our aim was, therefore, to obtain chromatographically pure phenylmethylsulfonyl-subtilisin rather than to purify subtilisin.

*Chromatography of Phenylmethylsulfonyl-subtilisin.* Phenylmethylsulfonyl-subtilisin was prepared by treating the chromatographically homogeneous fraction of subtilisin with phenylmethylsulfonyl fluoride (Polgar and Bender, 1967). Subsequently, it was chromatographed at pH 6.5 as described for subtilisin. The rechromatography of phenylmethylsulfonyl-subtilisin resulted in a virtually complete binding to the ion-exchange column, in contrast to subtilisin. This indicates the absence of denaturation of the modified enzyme and suggests that denaturation of the active enzyme is due to autodigestion.

Phenylmethylsulfonyl-subtilisin appeared in the same chromatographic fraction as subtilisin. This was demonstrated by the chromatography of a 9:1 mixture of phenylmethylsulfonyl-subtilisin and native enzyme: the ratio of enzymatic activity to protein concentration was constant in all fractions.

*Activity and Chromatography of Thiol-subtilisin.* Purified phenylmethylsulfonyl-subtilisin was used for the preparation of thiol-subtilisin. To displace the phenylmethylsulfonyl group from the carbon atom of the serine residue at the active site, thiolacetate ion was employed as described previously (Polgar and Bender, 1967). Thiol group (1.00 ± 0.05 equiv) could be determined in thiolsubtilisin obtained from the purified phenylmethylsulfonyl enzyme by Boyer's (1954) *p*-mercuribenzoate titration method. This preparation should be free of extraneous hydrolase activity, with the possible exception of some subtilisin which could be regenerated from phenylmethylsulfonyl-subtilisin. Using different preparations, we found that in the presence of *p*-mercuribenzoate the hydrolysis of *p*-nitrophenyl acetate was completely inhibited, while that of *p*-nitrophenyl *N*-benzyloxycarbonylglycinate was only inhibited 40–70%. Based on the activity tested by *p*-nitrophenyl *N*-benzyloxycarbonylglycinate, the thiol derivative may contain at most 0.2% native enzyme.

We attempted to separate thiol-subtilisin from a possible contamination with the native enzyme. A Carboxymethylcellulose column equilibrated with 0.01 M phosphate buffer (pH 6.5) was used to study the chromatographic behavior of the thiol enzyme. In marked contrast to subtilisin, the thiol derivative was not firmly bound to the ion-exchange column. Under conditions when carboxymethylcellulose bound more than 99% subtilisin, thiol-subtilisin passed through the column and showed a considerable tailing. Thus the thiol enzyme purified by chromatography should be free of subtilisin. Nevertheless, the initial rate of the hydrolysis of *p*-nitrophenyl *N*-benzyloxycarbonylglycinate with this preparation could only be inhibited 60–70% with *p*-mercuribenzoate as seen in Table I. This indicates that thiol-subtilisin itself is responsible for the activities both in the presence and in the absence of *p*-mercuribenzoate. One can argue, however, that the binding capacity of subtilisin may change in the presence of a large amount of the thiol derivative, and it may be eluted from the column even by 0.01 M phosphate buffer. Therefore, we added 13% subtilisin to the thiol enzyme

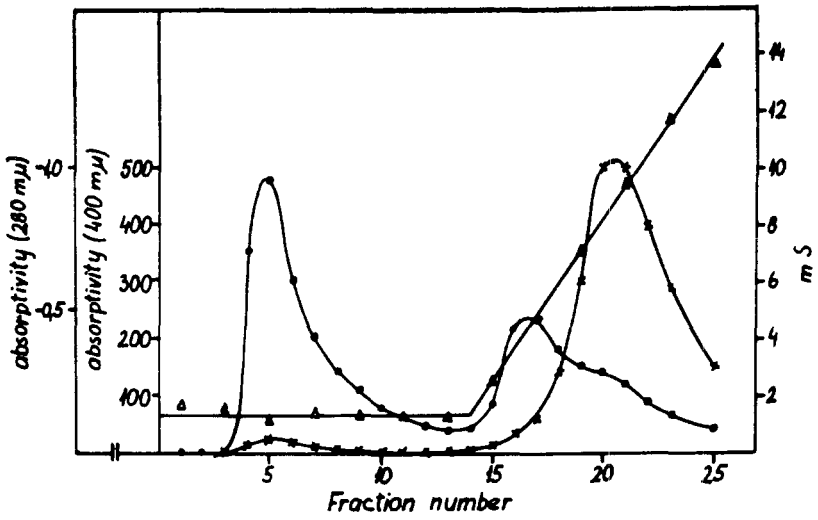


FIGURE 2: Chromatography of 87% thiol-subtilisin and 13% subtilisin at pH 6.5 using linear gradient elution with phosphate buffers. (○) Protein concentration at $E_{280}$; (+) enzymatic activity at $E_{400}$; (△) conductivity, m$S$.

and chromatographed this mixture. It is seen from Figure 2 that a high quantity of the protein appears in the first peak which shows a low specific activity characteristic of thiol-subtilisin. The increasing ionic strength displaces the tail fractions from the column resulting in a second protein peak. This is followed by a peak highly active toward *p*-nitrophenyl *N*-benzyloxycarbonylglycinate which is characteristic of the native enzyme. At the maximum of the curve related to the enzymatic activity, a small shoulder can be discerned on the second protein peak, which is due to added subtilisin.

The above chromatographic pattern shows that even in the presence of a high amount of thiol-subtilisin, subtilisin binds to carboxymethylcellulose in 0.01 M phosphate buffer (pH 6.5) and, therefore, a thiol-enzyme preparation free of subtilisin can be obtained. This observation lends further support to the idea that thiol-subtilisin is inherently endowed with an additional hydrolytic activity which is not inhibited by *p*-mercuribenzoate.

## Discussion

For preparation of thiol-subtilisin of high purity sufficiently pure subtilisin or phenylmethylsulfonyl-subtilisin would be required. In this paper we demonstrated by means of ion-exchange chromatography that a few per cent of the total hydrolytic activity of the commercial subtilisin preparation is due to a contamination with another hydrolase. Since autodigestion occurs under the conditions of the separation of the extra hydrolase activity from subtilisin, the inactive form of the enzyme, phenylmethylsulfonyl-subtilisin, was chromatographed in further studies. This derivative, which is routinely used for thiol-subtilisin synthesis, could be properly purified.

The displacement of the phenylmethylsulfonyl group from pure phenylmethylsulfonyl-subtilisin by thiolacetate ion results in the formation of acetyl-thiol-subtilisin which, in turn, hydrolyses to thiol-subtilisin. Since phenylmethylsulfonyl-subtilisin itself can slowly hydrolyze, a small amount of subtilisin may be present in the thiol-enzyme preparation. The binding of thiol-subtilisin to carboxymethylcellulose in 0.01 M phosphate buffer (pH 6.5) is significantly different from the binding of subtilisin; therefore, highly purified thiol-enzyme can be obtained. Namely, under the same conditions, subtilisin, phenylmethylsulfonyl-subtilisin, and any other hydrolytic enzyme that the purified phenylmethylsulfonyl-subtilisin eventually still contains bind to carboxymethylcellulose and only thiol-subtilisin passes through the column.

The chromatographed thiol-subtilisin which is free of subtilisin and of similar hydrolytic enzymes proves to be sufficiently pure when tested with *p*-nitrophenyl acetate. However, it appears to be inhomogeneous in the hydrolysis of *p*-nitrophenyl *N*-benzyloxycarbonylglycinate: SH reagents like *p*-mercuribenzoate and *N*-methylmaleimide do not inhibit more than 60–70% of its activity. As the same results are obtained with rechromatographed thiol-subtilisin, we conclude that in spite of the incomplete inhibition, all of the activity is due to thiol-subtilisin.

It could be argued that *p*-nitrophenyl *N*-benzyloxycarbonylglycinate may compete with *p*-mercuribenzoate because of its carbobenzyloxy group, and that this could result in an incomplete inhibition with *p*-nitrophenyl *N*-benzyloxycarbonylglycinate as the substrate. This is not likely, however, since S–Hg is a fairly stable bond. Moreover, this idea would not account for the fact that *N*-ethylmaleimide, which forms a covalent bond with the thiol group, decreases the initial rate to the same extent as *p*-mercuribenzoate does. The extra activity of thiol-subtilisin may be due to the catalysis by an imidazole group of the protein, as was suggested in the case of chymotrypsin (Kézdy and Bender, 1962) and myoglobin (Breslow and Gurd, 1962).

Because of the complex reactivity of thiol-subtilisin, *p*-nitrophenyl *N*-benzyloxycarbonylglycinate is not a

convenient substrate for kinetic investigations. However, it can be easily ascertained that the initial rate of the liberation of nitrophenolate ion by the SH group of thiol-subtilisin is about the same with *p*-nitrophenyl *N*-benzyloxycarbonylglycinate as with *p*-nitrophenyl acetate. It is important to note that thiol-subtilisin greatly differs from subtilisin because for the latter enzyme at pH 7.0 the $k_{cat}$ for *p*-nitrophenyl *N*-benzyloxycarbonylglycinate (11.2 $sec^{-1}$) is about 100 times higher than the $k_{cat}$ for *p*-nitrophenyl acetate (0.105 $sec^{-1}$). The decrease in the rate of hydrolysis of *p*-nitrophenyl *N*-benzyloxycarbonylglycinate is approximately three orders of magnitude while we have previously found a 30-fold decrease for the $k_{cat}$ of *p*-nitrophenyl acetate hydrolysis (Polgar and Bender, 1967). Neet and Koshland only measured a threefold decrease in the same reaction (Neet and Koshland, 1966). In their experiments the assay conditions for different substrates were specified as phosphate or Tris buffer. If Tris buffer was chosen for *p*-nitrophenyl acetate hydrolysis the tenfold difference between the two measurements may be conceivable since thiol esters are much more sensitive toward nitrogen nucleophiles than the oxygen esters are (Kosower, 1962) and so the amino group of the Tris buffer will compete with water in attacking the acetyl thiol enzyme. In fact, this phenomenon was found to be the case with papain (Brubacher and Bender, 1966) and thiol-subtilisin (Polgar and Bender, 1967).

Since deacylation is the rate-limiting step of the hydrolysis of nitrophenyl esters by proteolytic enzymes, *p*-nitrophenyl *N*-benzyloxycarbonylglycinate is suitable for studying the deacylation of subtilisin in a reaction with a highly specific substrate. If thiol-subtilisin can only react with substrates bearing a good leaving group, as suggested earlier (Polgar and Bender, 1967), *p*-nitrophenyl *N*-benzyloxycarbonylglycinate is of a particular interest since it can be regarded both as an activated and a specific ester. By treating *p*-nitrophenyl *N*-benzyloxycarbonylglycinate with the thiol enzyme it does form a specific acyl thiol enzyme, which may not be formed with simple specific esters.

In a study of this reaction we have found that the more specific substrate for subtilisin is by no means the better substrate for the thiol derivative even though an acyl thiol enzyme apparently can be formed. The reaction of *p*-nitrophenyl *N*-benzyloxycarbonylglycinate with thiol-subtilisin might show some of the features of an enzymatic hydrolysis since the rates of enzymatic hydrolysis of *p*-nitrophenyl *N*-benzyloxycarbonylglycinate and *p*-nitrophenyl acetate are of the same order of magnitude and the latter was recently shown to be an enzymatic process (Polgar and Bender, 1967). However, it is not possible to discuss meaningfully $k_{cat}$, acylation, and deacylation, because in the reaction of thiol-subtilisin with *p*-nitrophenyl *N*-benzyloxycarbonylglycinate there is a considerable extra activity which is not inhibited by thiol reagents.

The difference in the chemical reactivity of hydroxyl and thiol groups cannot account for a decrease of three orders of magnitude in the rate of deacylation (Neet and Koshland, 1966). In order to explain the markedly reduced rate of catalysis, we have to assume that a subtle modification in the active center results in a distortion which interferes with the operation of the active center. Similar conclusions were drawn from the studies on thiol-alcalase (Polgar, 1968).

It is fairly unexpected that the replacement of one atom of a protein molecule for a similar atom alters the protein structure or its charge distribution in such a manner that it would be easily separated on an ion-exchange column, though a change in stereochemistry should be kept in mind. Indeed, we have shown that the bulky phenylmethylsulfonyl group does not interfere at all with the chromatographic behavior of subtilisin. On the other hand, thiol-subtilisin passes through the ion-exchange column while subtilisin binds 99% to the column. Such a pronounced change in the chromatographic properties can be ascribed to the alteration of the charge distribution or to the alteration of the steric structure of the protein. As to the latter, comparative studies of the optical rotatory dispersion or other simple physical parameters may hardly be meaningful since even an absolutely pure subtilisin, which is essential for these studies, would be altered in the course of the measurement because of its tendency to autodigestion. As to the difference in charge, replacement of the oxygen for a sulfur atom cannot affect the charge distribution of the protein. However, on the basis of kinetic investigations, we suggested (Polgar and Bender, 1967) that a hydrogen bond is formed between the thiol group and a kinetically important histidine residue of thiol-subtilisin, whereas a similar hydrogen bond between the imidazole and the hydroxyl groups does not exist in subtilisin. Hydrogen-bond formation would cause the loss of one positive charge in the thiol enzyme, which, in turn would result in a requirement for a more acidic medium for the binding of thiol-subtilisin to carboxymethylcellulose than for the binding of subtilisin. Consequently, the separation of subtilisin from its thiol derivative during chromatography offers physical–chemical evidence to support our previous assumption concerning hydrogen-bond formation in thiol-subtilisin (Polgar and Bender, 1967).

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# Further Observations on the Chemical Nature of Rubredoxin from *Clostridium pasteurianum**

Walter Lovenberg and William M. Williams

With the Technical Assistance of Eleanor C. Bruckwick

ABSTRACT: The nature of the iron binding of rubredoxin undoubtedly accounts for the redox properties of the protein. The half-cysteine residues in the protein appear to form four of the ligands of the iron. This conclusion was derived from measuring the reactivity of the protein toward sulfhydryl reagents (iodoacetate and mercurials) and the finding that 2-mercaptoethanol was required for an $^{59}Fe$ exchange reaction and for reconstitution of the apoprotein. It was also observed that rubredoxin could undergo its complete redox cycle in the crystalline state without apparent damage to the crystal. Finally, an improved technique for the isolation of rubredoxin from *Clostridium pasteurianum* is described.

Rubredoxin is a nonheme iron protein which has been found to be present in many clostridial species (Lovenberg and Sobel, 1965; Stadtman, 1965; Mayhew and Peel, 1966; LeGall and Dragoni, 1966). While no unique physiological role has been established for this clostridial protein, a protein containing a similar iron chromophore has been isolated and shown to participate in hydrocarbon oxidation in *Pseudomonas oleovorans* (Peterson and Coon, 1968). Previous studies (Lovenberg and Sobel, 1965) in this laboratory have shown the clostridial protein to have a molecular weight of about 6000 and to contain one atom of iron. Rubredoxin has absorption maxima at 490, 380, and 280 mμ, shows optical activity (Lovenberg, 1966), and undergoes reversible one-electron oxidation and reduction with a redox potential of about −0.057 V (Lovenberg and Sobel, 1965). Rubredoxin exhibits an electron spin resonance signal at $g = 4.3$ in the oxidized state but no observable signal in the reduced state (Lovenberg, 1966). Recently the complete amino acid sequences of rubredoxin from *Micrococcus aerogenes* and *Peptostreptococcus elsdenii* have been reported (Bachmayer *et al.*, 1967a, 1968a).

It has been suggested (Lovenberg, 1966; Bachmayer *et al.*, 1967a,b) that the cysteine residues may participate in the iron binding properties of this protein. The current study was undertaken to further clarify the role of the cysteines and to examine possible molecular changes which occur during the oxidation–reduction cycle of the protein. In addition an improved method for isolation of the protein is reported.

* From the Experimental Therapeutics Branch, National Heart Institute, Bethesda, Maryland. *Received June 24, 1968.*

## Materials

[1-$^{14}$C]Iodoacetic acid (1.4 μCi/μmole), $^{59}FeCl_3$ (2.9 mCi/μmole), and omnifluor scintillator solution were obtained from New England Nuclear Corp. The iodoacetic acid (100 μCi) was mixed with 1.5 g of unlabeled material dissolved in a minimal volume of chloroform and recrystallized. The white crystalline material had a specific activity of $2.22 \times 10^4$ cpm/μmole under standard counting conditions. Other reagents were the best grades available from commercial sources. *Clostridium pasteurianum* was grown on a medium containing $(NH_4)_2SO_4$ as its sole nitrogen source as described previously (Lovenberg *et al.*, 1963).

*Rubredoxin* was isolated from frozen cells (Lovenberg and Sobel, 1965) through the second DEAE-cellulose step. In this step the 90% saturated $(NH_4)_2SO_4$ solution is applied to a DEAE-cellulose column and the mixture of ferredoxin and rubredoxin is adsorbed to the ion-exchange resin. By use of a decreasing gradient of $(NH_4)_2SO_4$ concentration it is possible to obtain sequential elution of these two proteins. As seen in Figure 1 when approximately 1 mg each of the two highly purified proteins was applied as a mixture to a $1.0 \times 10$ cm DEAE-cellulose in a 90% saturated $(NH_4)_2SO_4$ solution and a linear gradient of 100% saturated $(NH_4)_2SO_4$ to water applied, rubredoxin (peak A) was eluted starting at about 45% saturation and ferredoxin (peak B) at about 35% saturation. Following this step the rubredoxin was concentrated by increasing the $(NH_4)_2SO_4$ concentration to about 80% and readsorbing on a small DEAE-cellulose column. The rubredoxin was eluted from the column with 0.8 M $Cl^-$ buffer (0.65 M NaCl and 0.15 M Tris-HCl, pH 7.3). The rubredoxin was next de-