generation of thiocystine, CySSSCy, from the reaction of cystine with the unstable cysteine hydropersulfide has been noted.[14] The latter is obtained enzymatically by the action of cysteine desulfhydrase on cystine.[15]

The reconstitution of parsley apoferredoxin in the presence of rhodanese (thiosulfate–cyanide sulfur transferase), dithiothreitol, and thiosulfate[16] suggests that the sulfur transferase activity of rhodanese also could be involved in ferredoxin biosynthesis. The equilibrium (eq 1) that describes the "sulfur-rich" rhodanese ($E{\cdot}S_2$) action on dihydrolipoate has been reported.[17]

$$E{\cdot}S_2 + 2\text{lip}(SH)_2 \rightleftarrows 2\text{lip}S_2 + 2HS^- + 2H^+ + E \quad (1)$$

In the course of formation of lipoate, $\text{lip}S_2$, a chromophore was observed and assumed to be an organic persulfide intermediate. Whether this intermediate, with a hydropersulfide functional group, lip(SH)(SSH), undergoes further reaction with lipoate (eq 2) with formation of a cyclic lipoate trisulfide remains to be established. Such a reaction, however, would be analogous to the reaction reported to occur between CySSH and CySSCy[14] (eq 3). A suggestion is advanced, that linear or cyclic trisulfides, upon reduction, may serve as sources for inorganic sulfide. A synthesis of cyclic trithiothreitol was undertaken to examine the ability of this molecule to act as an oxidizing agent for the $[Fe(SC_6H_5)_4]^{2-}$ complex. Trithiothreitol[18] reacts readily with $[Fe(SC_6H_5)_4]^{2-}$ in DMF to form $[Fe_2S_2(SC_6H_5)_4]^{2-}$, which was obtained in good yields and characterized by comparison with an "authentic" sample.[7]

$$\text{lip}(SH)(SSH) + \text{lip}S_2 \rightarrow \text{lip}S_3 + \text{lip}(SH)_2 \quad (2)$$

$$\text{CySSH} + \text{CySSCy} \rightleftarrows \text{CySSSCy} + \text{CySH} \quad (3)$$

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Supplementary Material Available. Observed structure factors for $[(C_6H_5)_4P]_2Fe_2S_{12}$ (9 pages). Ordering information is given on any current masthead page.

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D. Coucouvanis,* D. Swenson
P. Stremple, N. C. Baenziger
Department of Chemistry, University of Iowa
Iowa City, Iowa 52242
Received January 22, 1979

Peptide Synthesis in Water and the Use of Immobilized Carboxypeptidase Y for Deprotection

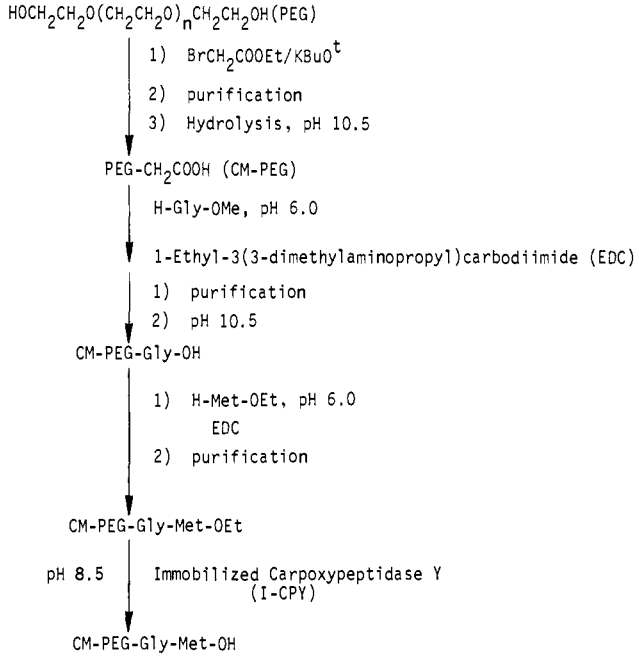
Sir:

We report here a method for peptide synthesis in aqueous systems in which coupling and deblocking are carried out at pH 6 and 8.5, respectively (Scheme I). Water-soluble carbodiimide and amino acid ethyl esters are used to elongate the chain from the carboxyl end (Scheme II). The removal of the ethyl ester blocking group is accomplished by treatment with immobilized carboxypeptidase Y (CPY) at pH 8.5 and room temperature. The bound enzyme is removed by filtration and the filtrate used directly for the addition of the next amino acid. Peptides were grown on carboxymethyl-poly(ethylene glycol)-glycylmethionine (CM-PEG-Gly-Met). The use of this handle provides solubility for the growing chain and permits facile release of finished peptide using CNBr.[1] The advantages of a polymeric handle have been pointed out by Mutter et al., who used underivatized PEG in synthesis of peptides by extension of the amino terminus in organic solvents.[2] The preparation of chemically and optically pure H-Leu-Phe-Leu-OEt is illustrated below.

CPY is an exopeptidase from yeast with a very broad specificity.[3] The enzyme also has esterase activity. An important point for this study is that the pH optima for the peptidase and esterase activities are quite different. In preliminary studies, we demonstrated the absence of peptidase activity in the time required to deblock Z-Leu-Phe-OEt. Immobilization of the enzyme on CL-Sepharose does not appear to perturb the pH profiles of the enzyme.[5] It is also significant that a D-amino acid at the C terminus or the penultimate position prevents ester hydrolysis by CPY. This feature assures optical purity of the final product.

PEG (14 g, mol wt 6000–7000, MCB) and potassium *tert*-butoxide (10 g, Aldrich) were dissolved in *tert*-butyl alcohol (150 mL) by warming to 40 °C. Ethyl bromoacetate (5 mL) was added over a period of 10 min. After we stirred the solution for 2 h, the solvent was evaporated. The residue was dissolved in 100 mL of 1 N NaOH. After 2 h at room temperature, the pH of the mixture was adjusted to 2. The CM-PEG was extracted into $CHCl_3$ (two 200-mL portions). The organic extract was washed with water and dried with anhydrous

Scheme I. Preparation of the Handle



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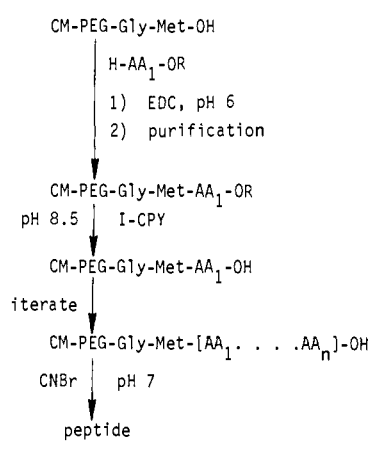
Na_2SO_4. Evaporation of the solvent yielded 12 g of CM-PEG.

Gly-OEt·HCl (1.4 g) and CM-PEG (3.4 g) were dissolved in 35 mL of water. The pH was adjusted to 6 with triethylamine. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (2.0 g) was added, and the pH was maintained by automatic addition of 0.1 N HCl. After 3 h at room temperature, the reaction mixture was acidified to pH 2 and the product was extracted into 250 mL of $CHCl_3$. The organic layer was washed with 1 N HCl and water. After removal of the $CHCl_3$, the CM-PEG-Gly-OEt was saponified at pH 10.5 using a pH stat and 0.1 N NaOH. After acidification and extraction, the product (3.3 g) had 100 nmol of glycine/mg according to amino acid analysis.

Met-OEt·HCl was reacted in a manner analogous to Gly-OEt. CM-PEG-Gly-Met-OEt was deblocked by treatment with immobilized CPY at pH 8.5. About 30 ATEE units of enzyme was used.[3] The enzyme was immobilized on CL-Sepharose as previously described,[5] or by adsorption on immobilized concanavalin A with glutaraldehyde cross-linking.[6] Deblocking of CM-PEG-Gly-Met-OEt took 5 h. However, as the chain length is increased, the deblocking time was dramatically reduced, probably as a result of increased subsite binding. CM-PEG-Gly-Met-Leu-Phe-Leu-OEt was prepared by successive addition of Leu-OEt, Phe-OEt, and Leu-OEt as described above. Starting with 1.75 g of CM-PEG-Gly-Met-OEt produced 1.50 g of CM-PEG-Gly-Met-Leu-Phe-Leu-OEt. Amino acid analyses, performed at each step using a hydrolysate of the CM-PEG-peptide, indicated that coupling and deblocking went to completion. Hydrolysis was performed according to Westall and Hesser.[7]

Release of the peptide was accomplished by treatment of CM-PEG-Gly-Met-peptide with CNBr at room temperature. CM-PEG-Gly-Met-peptide (180 mg) was dissolved in 1 mL of distilled water. To this 0.018 mL of a CNBr stock solution (0.1 M in acetonitrile) was added. The mixture was lyophilized after 1 h. Water (1 mL) was added, the pH of the mixture was adjusted to 2, and lyophilization was repeated. The residue was dissolved in $NaHCO_3$ buffer (pH 8.5), and the solution was extracted with EtOAc. CNBr treatment of 180 mg of CM-PEG-Gly-Met-Leu-Phe-Leu-OEt yielded 4.5 mg of peptide (64% based on glycine content of the handle). The product, H-Leu-Phe-Leu-OEt, was identical with a sample of authentic peptide as judged by TLC: R_f 0.79 (*n*-BuOH-AcOH-H_2O 4:1:5) and R_f 0.85 (*n*-BuOH-AcOH-H_2O-pyridine 15:3:10:12). The optical rotation of our product was similar to that of the standard: $[\alpha]^{25}_D$ −0.45° (standard, 1% in EtOH) and −0.42 (product, 1% in EtOH). The amino acid analyses of the released peptide showed the following: sample 1, 20.9 nmol of Leu and 10.4 nmol of Phe; sample 2, 45.8 nmol of Leu and 21.9 nmol of Phe; sample 3 (hydrolyzed with immobilized aminopeptidase M[8]), 27.0 nmol of Leu and 13.5 nmol of Phe.

Scheme II. Chain Elongation and Release



We demonstrated the absence of peptidase activity during the time required for complete deblocking of the ester, Z-Leu-Phe-OEt. As an additional test, we checked for peptidase activity on a very good CPY substrate attached to PEG. After complete deblocking of CM-PEG-Phe-Met-Tyr-OEt (~20 min, pH 8.5, 25 °C), a sample corresponding to 1000 nmol of tyrosine was applied to the amino acid analyzer. An insignificant amount of free tyrosine was detected (about 0.1%).

The question of racemization arises when extension of the C terminus of the peptide chain is chosen as the approach to sequential synthesis. The generally accepted mechanism for racemization involves the deprotonation of an oxazolinone intermediate.[9] We reasoned that this deprotonation would not be significant at pH 6 in water. Z-Leu-Phe-Leu-OEt made by our method had an optical rotation identical with that of a sample made by the excess mixed anhydride method. Our peptides are chemically homogeneous at every stage, indicating that complete deprotection occurred. Base uptake during the deprotection step also matched the predicted amount. Also, agreement of amino acid ratios between analyses on samples of H-Leu-Phe-Leu-OEt prepared by acid and enzymatic hydrolysis strongly suggests that our method yields optically pure peptides.

For a more sensitive racemization test we adapted the method of Kitada and Fujino,[10] which is based on the separation of dipeptide diastereomers.[11] With a 60 × 0.9 cm column of Beckman AA-15 resin, L-Leu-L-Leu (60 min) was well separated from D-Leu-L-Leu (75 min). The conditions were as follows: flow rate, 1 mL/min; column temperature, 55 °C; buffer, 0.35 M citrate (pH 5.25). The method is capable of detecting 1 nmol of D-L isomer in 200 nmol of peptide. CM-PEG-Phe-Leu-Leu-OH was prepared as described above. The dipeptide was released using α-chymotrypsin. Also, two separate batches of PEG-Phe-Met-Leu-Leu-OH were prepared and cleaved with CNBr according to ref 9. The maximum extent of racemization was 0.7% out of nine analyses involving three distinct batches of peptide.

Another question is whether or not deblocking with CPY is applicable in the synthesis of peptides that involve side-chain protection. We have synthesized H-Ala-Ala-Cys(ACM)-Lys(Z)-OH by the method detailed above. Deprotection and coupling went to completion at each stage as judged by TLC and amino acid analyses. About 320 mg of PEG-Gly-Met-Ala-Ala-Cys(ACM)-Lys(Z)-OH was obtained starting with 400 mg of PEG-Gly-Met-OEt. The peptide was released as described above (42% yield after extraction). The analytical data of the released peptide follow (amino acid analysis, nanomoles): Ala, 88.4; Cys(ACM), 42.2; Lys_1, 44.4; TLC, R_f 0.52 (*n*-BuOH-AcOH-H_2O, 4:1:5; silica gel).

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Garfield P. Royer,* G. M. Anantharmaiah
Department of Biochemistry, The Ohio State University
Columbus, Ohio 43210
Received June 12, 1978

Kinetics of CO Binding to Fe(TPP)(Im) and Fe(TPP)(Im$^-$): Evidence Regarding Protein Control of Heme Reactivity

Sir:

Protein structure modulation of the reactivity of a heme prosthetic group has been a focus for numerous investigations.[1] For example, ligand binding kinetics of hemoglobin are affected by protein conformation.[1a,2] The CO on-rate for the low-affinity T state is ~20–60-fold less than that for the high-affinity R state.[3] One plausible mechanism of this modulation has been advanced by Peisach and collaborators.[4] It is generally proposed that a protein can induce changes in heme reactivity through structural changes at the proximal imidazole. For hemoglobin, in the low-affinity state the proximal histidine is thought to be in its neutral form with a proton on N-1. The high-affinity form is considered to have a strong hydrogen bond to that proton, which in the limit might be thought of as corresponding to a deprotonated imidazole as the sixth ligand. Such a mechanism also has been invoked to discuss the electronic structure of cytochromes *c* from different organisms,[5] and the crystal structures of a number of hemoproteins.[6]

Prompted by our previous work on the mechanisms of heme–protein interactions,[5,7] we now have tested the relation of heme reactivity and the imidazole protonation state by the direct comparison of the CO binding rate, for a five-coordinate ferrous porphyrin model with neutral imidazole as fifth ligand, Fe(P)(Im),[8] with that for the model with deprotonated imidazole as the fifth ligand, Fe(P)(Im$^-$). Deprotonation can indeed alter the CO binding rates to a degree in excess of the difference in binding rates to the T and R states of Hb. However, the difference is in the opposite sense to that which would normally be expected: deprotonation *decreases* the rate of CO binding.

Kinetic measurements were made by monitoring absorbance changes after flash photolysis of an Fe(TPP)(B)(CO), B = Im, Im$^-$, solution,[9-12] using a computer-interfaced apparatus of conventional design.[13] The Fe(TPP)(Im)(CO) complex is highly photolabile and the photoproduct rebinds CO with a rate constant, $k_{obsd} \propto [CO]$. However, the difference spectrum of Fe(TPP)(Im)(CO) and the product obtained immediately after the flash (~20 μs) does not correspond to the formation of Fe(TPP)(Im), which should have a Soret maximum at ~435 nm.[11] Rather, the kinetic difference spectrum is the same as the static difference spectrum [Fe(TPP)(Im)$_2$] − [Fe(TPP)(Im)(CO)], obtained by direct subtraction of the absorbance spectra of the appropriate complexes, which is also presented in Figure 1A. This indicates that a second Im is bound within the flash lifetime. Therefore the overall stoichiometry of the CO rebinding reaction as observed in the regeneration of Fe(TPP)(Im)(CO) after the photolysis flash is ligand replacement

$$\text{Fe(TPP)(Im)}_2 + \text{CO} \rightarrow \text{Fe(TPP)(Im)(CO)} + \text{Im}$$

and not the ligand addition reaction

$$\text{Fe(TPP)(Im)} + \text{CO} \xrightarrow{k_5} \text{Fe(TPP)(Im)(CO)}$$

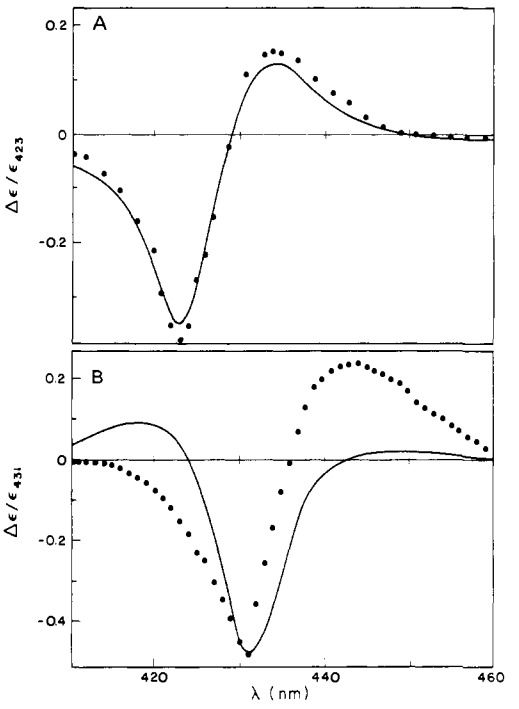
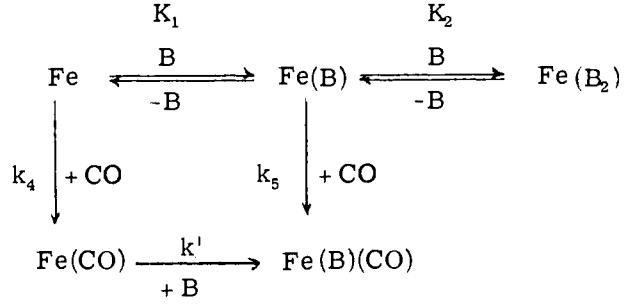


Figure 1. (A) Static difference spectrum, Fe(TPP)(Im)$_2$–Fe(TPP)(Im)(CO), —; kinetic difference spectrum after flash photolysis of Fe(TPP)(Im)(CO), ···. (B) Static difference spectrum, Fe(TPP)(Im$^-$)$_2$–Fe(TPP)(Im$^-$)(CO), —; kinetic difference spectrum after flash photolysis of Fe(TPP)(Im$^-$)(CO), ···.

Scheme I



which would correspond to the CO binding reaction by Hb.

This observation is expected from previous studies, such as those of Traylor;[14] binding of CO to an iron(II) porphyrin in the presence of excess base (B) can usually be described in terms of a preequilibrium between the Fe(B)$_n$ (n = 0, 1, 2),[11,14] where we have suppressed the porphyrin abbreviation (Scheme I). Here K_1 and K_2 are the measured static binding constants, and k_4 and k_5 are the second-order CO binding rates, of which k_5 is the quantity of interest; the reverse reaction, loss of CO, can be neglected on our time scale. When comparing rates for different bases, we will keep track by writing k_5(B).

Under the conditions of our experiments, the results of White et al.[14c] show that CO addition is rate limiting, and that the ligand rebinding rate obeys an equation

$$k_{obsd}/[\text{CO}] = k_4/\Sigma + k_5(K_1[\text{B}]/\Sigma) \qquad (1)$$

where $\Sigma = 1 + K_1[\text{B}] + K_1K_2[\text{B}]^2$. The equilibrium constants for binding Im by Fe(TPP) are known: $K_1 = 8.8 \times 10^3$ M^{-1} and $K_2 = 7.9 \times 10^4$ M^{-1}.[11] We performed a series of rate measurements at constant [CO] and with varying [Im], and, from the plot of $k_{obsd}\Sigma/[\text{CO}] = k_4 + k_5K_1[\text{B}]$ vs. [B] (Figure 2), we obtain k_4 from the interecept and k_5 from the slope (Table I).

The Fe(TPP)(Im$^-$)(CO) complex is also photolabile, and

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