Oxygen Equilibrium Properties of Highly Purified Human Adult Hemoglobin Cross-Linked between $82\beta_1$ and $82\beta_2$ Lysyl Residues by Bis(3,5-dibromosalicyl) Fumarate

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ABSTRACT: We investigated oxygen equilibrium properties of highly purified human adult hemoglobin cross-linked between lysine-$82\beta_1$ and lysine-$82\beta_2$ by a fumaryl group, which is prepared by reaction of the CO form with bis(3,5-dibromosalicyl) fumarate. The cross-linked hemoglobin preparation isolated by the previous purification method, namely, gel filtration in the presence of 1 M $MgCl_2$ followed by ion-exchange chromatography, was found to be contaminated with about 20% of an electrophoretically silent impurity that shows remarkably high affinity for oxygen. This impurity was separated from the desired cross-linked hemoglobin by a newly developed purification method, which utilizes a difference between the authentic hemoglobin and the impurity in reactivity of the sulfhydryl groups of cysteine-93β toward *N*-ethylmaleimide under a deoxygenated condition. After this purification procedure, the oxygen equilibrium properties of purified cross-linked hemoglobin in the absence of organic phosphate became very similar to those of unmodified hemoglobin with respect to oxygen affinity, cooperativity, and the alkaline Bohr effect. The functional similarity between the cross-linked hemoglobin and unmodified hemoglobin allows us to utilize this cross-linking for preparing asymmetric hybrid hemoglobin tetramers, which are particularly useful as intermediately liganded models. Previous studies on this type of cross-linked hemoglobin should be subject to reexamination due to the considerable amount of the impurity.

A number of methods for cross-linking of hemoglobin (Hb)[1] have been developed for their clinical usefulness (Zaugg et al., 1975; Walder et al., 1977, 1979; Fantl et al., 1987; Kavanaugh et al., 1988; Bucci et al., 1989). One of the most extensively investigated compounds for cross-linking is the bifunctional acylating agent bis(3,5-dibromosalicyl) fumarate. Walder et al. (1980) have shown that this agent, when reacted with oxyHb, specifically cross-links Lys-$82\beta_1$ and Lys-$82\beta_2$ at the DPG binding site. The first report on the oxygen equilibrium properties of XL82β-Hb by Walder et al. (1980) showed a high affinity for oxygen at the low saturation range and somewhat reduced cooperativity compared to native Hb A. They suggested that such functional differences between XL82β-Hb and Hb A are ascribed to structural perturbations caused by the introduced fumaryl group, which is too short to span the distance between the two Lys82 residues of the β chains in deoxyHb (Walder et al., 1980).

Aside from the clinical application of these products, the cross-linking techniques have been successfully used to study the allosteric effects of the Hb molecule. The cross-linking between the two β chains by reacting oxyHb or CO-Hb with bis(3,5-dibromosalicyl) fumarate has been adopted for preparations of asymmetrically modified hybrid Hbs (Miura & Ho, 1982, 1984; Inubushi et al., 1986; Miura et al., 1987; Kitagishi et al., 1988; Kaminaka et al., 1989). Since the intermediate species in the course of oxygenation are present in low populations in a cooperative system, the hybrid molecules have been used as "models" for those intermediate species. Without cross-linking, the asymmetrically modified tetramers dissociate into the $\alpha\beta$ dimers, which then reassociate to form not only the former asymmetric tetramers but undesired symmetric ones. The cross-linking between the β chains by bis(3,5-dibromosalicyl) fumarate has the following advantages. First, the specificity in the reaction of this agent with oxyHb or CO-Hb is significantly high (Walder et al., 1979; Miura & Ho, 1982; Kikugawa et al., 1982). Second, the specific site of the cross-linking is located on the molecular 2-fold rotation axis of Hb, so that the cross-linking does not introduce any asymmetric distortion into the Hb tetramer (Walder et al., 1980). Third, the X-ray crystallographic study on deoxyXL82β-Hb by Walder et al. (1980) showed that this cross-linking causes only minor (symmetric) perturbations on the structure of the Hb molecule. Thus, it is likely that XL82β-Hb substantially preserves the nature of unmodified Hb molecule.

According to recent determination and analysis of oxygen equilibrium curves of XL82β-Hb (Miura et al., 1987), the relative magnitudes of K_1, K_2, K_3, and K_4 [K_i (i = 1–4) denotes the association equilibrium constants for the ith step of oxygenation] of XL82β-Hb were in contrast to those of Hb A. The relative magnitudes of K_i values of XL82β-Hb were in

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[1] Abbreviations: Hb, hemoglobin; Hb A, human adult hemoglobin; XL82β-Hb, hemoglobin cross-linked between Lys-$82\beta_1$ and Lys-$82\beta_2$ residues by a fumaryl group; DPG, 2,3-diphosphoglycerate; 4-PDS, 4,4′-dithiopyridine; Tris, tris(hydroxymethyl)aminomethane; Bistris, 2-[bis(2-hydroxymethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Mops, 3-*N*-(morpholino)propanesulfonic acid; NEM, *N*-ethylmaleimide; NES, sulfhydryl group of Cys-93β specifically reacted with *N*-ethylmaleimide; Cys$^+$, sulfhydryl group of Cys-93β specifically reacted with cystamine; IHP, inositol hexaphosphate; NFPLP, 2-nor-2-formylpyridoxal 5′-phosphate; HPLC, high-performance liquid chromatography.

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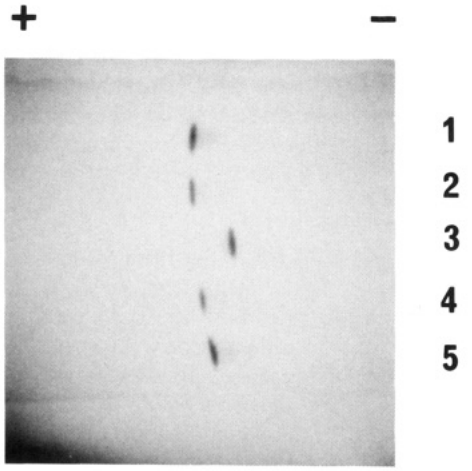

FIGURE 1: Isoelectric focusing of XL82β-Hb and Hb A in CO forms. Lanes 1 and 5, XL82β-Hb purified by the previous method; lanes 2 and 4, XL82β-Hb purified by the present method; lane 3, Hb A.

the order $K_2 \leq K_3 < K_1 \leq K_4$ whereas those of Hb A were in the order $K_1 \leq K_2 < K_3 \leq K_4$. A reversal of the order of K_1 and K_3 gave rise to peculiarly shaped Hill plots for oxygenation of XL82β-Hb. If such marked functional differences between XL82β-Hb and native Hb A actually existed, the β_1–β_2 cross-linking would lose its adequacy for studies on the cross-linked asymmetric hybrid Hbs.

In the present paper we present evidence that previous XL82β-Hb samples were contaminated with about 20% of a high-oxygen-affinity impurity that cochromatographed with XL82β-Hb in earlier studies. We have developed a unique purification method, in which such a chromatographically silent impurity is removed by a difference in reactivity of the sulfhydryl groups of cysteine-93β under a deoxygenated condition. Accurate oxygen equilibrium curves of the purified XL82β-Hb samples proved to be very similar to those of native Hb A, in contrast with previous observations.

Experimental Procedures

Preparation of Human Adult Hemoglobin. Human blood hemolysate was prepared according to Kilmartin and Rossi-Bernardi (1971). The CO-form hemolysate was deionized by passage through a column of Amberlite MB-3 (Rohm & Hass). Then, the deionized hemolysate was applied to a column of CM52 cellulose (Whatmann) equilibrated with 0.0028 M phosphate buffer, pH 7.5 (1.8-mL column bed volume is necessary for 1 g of Hb). Hb A_2 free Hb was eluted from this column, as the total amount of Hb A was much larger than the exchange capacity of the column. For the next step of purification, 2 M Tris was added to the Hb A_2 free Hb to attain the final concentration of 0.01 M. Then, the Hb A_2 free Hb was applied to a column of DE52 cellulose (Whatmann) equilibrated with 0.005 M Tris and 0.001 M acetate buffer (10-mL column bed volume is necessary for 1 g of Hb). Hb A was eluted by passage of 2.2 column volumes of 0.05 M Tris and 0.028 M acetate buffer. The substantial part of Hb A_1 components was removed by this procedure. The purified Hb appeared as a single band on analytical isoelectric focusing using Pharmalyte pH 6–8 (Pharmacia) (lane 3 in Figure 1). All the procedures were carried out at 4 °C under a CO gas atmosphere.

Preparation of Cross-Linked Hemoglobin (XL82β-Hb). Bis(3,5-dibromosalicyl) fumarate was synthesized according to Walder et al. (1979) and recrystallized twice from ethanol. A mixture of CO-Hb and a stoichiometric amount of bis-(3,5-dibromosalicyl) fumarate in 0.05 M MOPS–NaOH buffer, pH 7.1, was incubated for 2 h at 37 °C under a CO gas atmosphere. After passage of the reaction mixture through a column of Sephadex G-25 (Pharmacia), the un-cross-linked derivatives were separated by gel-filtration column chromatography on an Ultrogel AcA44 column (LKB) in the presence of 1 M $MgCl_2$ as described by Miura and Ho (1982). The fraction of cross-linked tetramer was applied to a column of CM52 cellulose equilibrated with 0.01 M phosphate buffer, pH 6.85. The main peak eluted with a linear gradient of 0.01 M phosphate buffer, pH 6.95, and 0.015 M phosphate buffer, pH 7.38, was collected. The product appeared as a single band on analytical isoelectric focusing (lanes 1 and 5 in Figure 1). However, a kinetic measurement of sulfhydryl reactivity of Cys-93β with 4-PDS under a deoxygenated condition revealed about 20% impurity with high reactivity.

This impurity was removed by utilizing its much increased sulfhydryl reactivity as follows. Typically, 400 mg of CO-form preparation containing cross-linked Hb in 6 mL of 0.1 M phosphate buffer, pH 7.2, was converted to the oxy form by illumination under a continuous steam of pure oxygen as described by Imai (1982). The oxy sample was then deoxygenated in a tonometer with pure nitrogen but without adding sodium dithionite according to Kilmartin and Rossi-Bernardi (1971). A half-stoichiometric amount of N_2-saturated NEM solution was added to the sample by injection with a syringe under a nitrogen atmosphere, and the mixture allowed to stand for 90 min at 0 °C under a nitrogen atmosphere. The reaction of NEM was terminated by adding a 50-fold stoichiometric amount of N_2-saturated 2-mercaptoethanol (Wako, Osaka), and left at 0 °C for 30 min under a nitrogen atmosphere. The reaction mixture, after being flushed with CO, was passed through a Sephadex G-25 column equilibrated with 0.02 M Tris-HCl buffer, pH 8.2. A 50-fold stoichiometric amount of cystamine dihydrochloride, which reacts with the remaining free sulfhydryl groups of Hb, was added to the CO-Hb solution, and the mixture was allowed to stand for 1 h at 0 °C under a CO atmosphere. At this stage, there were three types of possible sulfhydryl modifications: (1) both the sulfhydryls were modified by two NEM molecules, i.e., $XL(\alpha\beta^{NES})(\alpha\beta^{NES})$, (2) one sulfhydryl was modified by NEM and the other cystamine, i.e., $XL(\alpha\beta^{NES})(\alpha\beta^{Cys+})$, and (3) both the sulfhydryls were modified by two cystamine molecules, i.e., $XL(\alpha\beta^{Cys+})(\alpha\beta^{Cys+})$. This mixture was passed through a Sephadex G-25 column equilibrated with 0.01 M phosphate buffer, pH 6.85, and then applied to a column of CM52 cellulose equilibrated with the same buffer. The Hb fractions were eluted with a linear gradient of 0.01 M phosphate buffer, pH 7.1, and 0.015 M phosphate buffer, pH 7.36 (Figure 2). The major peak, which corresponds to the cross-linked Hb reacted with two cystamine molecules (peak D in Figure 2), was eluted at pH 7.25 and then treated with dithiothreitol (5 mg/mL) in 0.1 M Tris-HCl buffer, pH 8.2, for 4 h at 0 °C under a CO atmosphere. After passage through a Sephadex G-25 column to remove the dithiothreitol, regeneration of the reactive sulfhydryl groups was confirmed by analytical isoelectric focusing. For precision oxygen equilibrium measurements we repeated this purification procedure twice.

Peptide Mapping. Heme-free native β chain was prepared as described previously (Shibayama et al., 1986). Purified XL82β-Hb in 0.01 M Tris-HCl buffer, pH 6.8, containing 1% (w/v) sodium dodecyl sulfate and 1% (v/v) 2-mercaptoethanol, was placed in a boiling water bath for several minutes to ensure complete denaturation. Separation of the α monomer and the cross-linked β dimer was performed by gel-filtration HPLC using a column of TSK gel G3000 SW (Toyo Soda) equilibrated with 0.05 M Bistris-HCl buffer, pH 7.0, containing

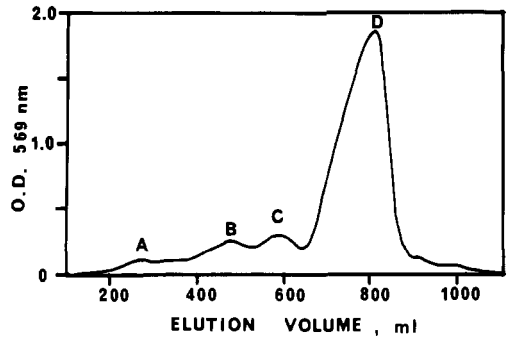

FIGURE 2: Elution profile of sulfhydryl-modified cross-linked Hbs separated by chromatography on a CM52 cellulose column. Sulfhydryl-modified cross-linked Hb samples (400 mg) were applied to a column of CM52 cellulose (3 × 20 cm), which was equilibrated with 0.01 M phosphate buffer, pH 6.85. The column was developed with a linear gradient of 0.01 M phosphate buffer, pH 7.1, and 0.015 M phosphate buffer, pH 7.36.

0.1% (w/v) sodium dodecyl sulfate and 0.2 M NaCl. The first effluent corresponding to the β dimer was collected.

The heme-free native β chain and the cross-linked β dimer were individually dialyzed against distilled water for 12 h and then equilibrated with 0.01 M Tris-HCl buffer, pH 8.2, with 0.1% (w/v) sodium dodecyl sulfate. They were digested with lysyl endopeptidase (E/S = 1/100) for 20 h at 37 °C. After a heat treatment at 100 °C for 5 min, the lysyl endopeptidase digests were further digested with trypsin (E/S = 1/30) for 24 h at 37 °C in the presence of 0.005 M $CaCl_2$.

The tryptic peptides were separated by reverse-phase HPLC on a High-Pore RP-304 column (Bio-Rad). Elution was carried out by using a gradient from 0% to 15% acetonitrile in 10 min, then to 50% acetonitrile in 140 min at a flow rate of 1 mL/min in the presence of 0.05% trifluoroacetic acid. The column effluent was monitored by absorbance at 215 nm.

Amino acid sequence was determined by automatic sequencing on a 470A gas-phase protein sequencer (Applied Biosystems).

Oxygen Equilibrium Measurements. Oxygen equilibrium curves were determined with an improved version (Imai, 1981) of an automatic oxygenation apparatus of Imai et al. (1970) interfaced to a microcomputer (Nippon Electric, Tokyo) for on-line data acquisition, storage, and analysis. The oxygen saturation was monitored at 560 nm with a Cary 118C spectrophotometer (Varian). In order to minimize the methemoglobin levels, catalase and superoxide dismutase were added to the Hb samples (Lynch et al., 1976; Winterbourn et al., 1976). In all cases, both the deoxygenation and reoxygenation curves agreed well with each other. Thus, the deoxygenation data were used for four-step Adair analysis as previously described (Imai & Yonetani, 1977). Overall oxygen affinity was expressed by partial pressure of oxygen at half-oxygen saturation, P_{50} (in millimeters of mercury: 1 mmHg = 133.3 Pa). Oxygen affinity at each step of oxygenation was expressed by the ith intrinsic Adair constants, K_i (i = 1–4) (in $mmHg^{-1}$). Cooperativity in oxygenation was expressed by the maximal slope of the Hill plot, n_{max}.

The methemoglobin contents were determined by the method of Evelyn and Malloy (1938) immediately after each measurement. In all cases, the methemoglobin contents in XL82β-Hb samples after measurement were less than 2.5%.

Reactivity of Sulfhydryl Groups with 4-PDS. The reaction of sulfhydryl groups of cysteine-93β with 4-PDS was measured by using a tonometer with a cuvette as described by Kilmartin et al. (1975). All measurements were carried out in 0.1 M phosphate buffer, pH 7.0, at 10 °C. The reactions were monitored at 324 nm with a Model U-3210 spectrophotometer (Hitachi, Tokyo). Visible absorption spectra were recorded before and after the 4-PDS kinetics to make sure complete deoxygenation was achieved and to determine the methemoglobin contents. In all cases, the methemoglobin contents after the kinetics measurements were less than 10%.

Results and Discussion

Purification of XL82β-Hb. After the cross-linking reaction, we followed the previous purification method, namely gel filtration in the presence of 1 M $MgCl_2$ followed by ion-exchange chromatography as described by Miura and Ho (1982). After this step of purification, analytical isoelectric focusing of this cross-linked Hb showed a single band that migrated toward the lower pH region compared to unmodified Hb A, due to the loss of two positive charges of two Lys-82β residues (lanes 1 and 5 in Figure 1). This observation is in full agreement with previous reports using the same purification method (Chatterjee et al., 1982; Miura & Ho, 1982). We measured an oxygen equilibrium curve of this cross-linked Hb at pH 7.47 in the presence of 0.1 M Cl^- at 25 °C. The Hill plot of the curve is presented in Figure 3A, together with that of native Hb A. The figure also includes a plot for XL82β-Hb[2] that was purified by preparative isoelectric focusing and gel filtration by Miura et al. (1987). Their data (broken line) agree with out data points for XL82β-Hb at low saturation range but slightly deviate at high saturation range. A feature common to our XL82β-Hb sample and theirs is a large shift of the lower portion of the Hill plot toward the left aparat from that of Hb A; this gives rise to the large K_1 values (Miura et al., 1987). The first report on oxygen equilibrium properties of XL82β-Hb purified by preparative isoelectric focusing (Walder et al., 1980) gave a significantly increased K_1 value. Moreover, Kikugawa et al. (1982) presented a similar result on XL82β-Hb S (Glu-6β → Val) purified by chromatography on DEAE-Sephadex. Therefore, it has been believed that the increased affinity for the first oxygen molecule is produced by the introduced cross-link bridge, which is too short to span between Lys-82β_1 and Lys-82β_2 in the deoxy quaternary structure, in agreement with the X-ray crystallographic results on deoxy XL82β-Hb (Walder et al., 1980; Chatterjee et al., 1982).

Since the low saturation range of oxygen equilibrium curves of Hb is sensitively affected by contamination of high-oxygen-affinity impurities, we suspected that this might be the case here. To examine the presence of such impurities, we measured the reactivity of sulfhydryl groups of Cys-93β toward 4-PDS. Since the reactivity of these sulfhydryl groups is very sensitive to the conformation of Hb, which governs the oxygen affinity (Perutz et al., 1974; Kilmartin et al., 1975), we expected that the impurity could be kinetically distinguished from authentic XL82β-Hb. The kinetic time courses for deoxyXL82β-Hb and native deoxyHb A are presented in Figure 4. The time course of deoxyXL82β-Hb is biphasic, while that of deoxyHb A is monophasic, the initial rate being strikingly increased in the former. This observation reveals chemical heterogeneity of the preparations by the previous purification method. It is known that Cys-93β reacts with sulfhydryl reagents more quickly in the oxy quaternary structure than in the deoxy quaternary structure (Perutz et al., 1974; Kilmartin et al., 1975). Thus, the fast component

[2] They presented the oxygen equilibrium data on the cross-linked Hb of which one $\alpha\beta$ dimer was derived from Hb A and the other was derived from Hb C (Glu-6β → Lys). It has been generally accepted that the oxygen equilibrium properties of Hb C are identical with those of Hb A.

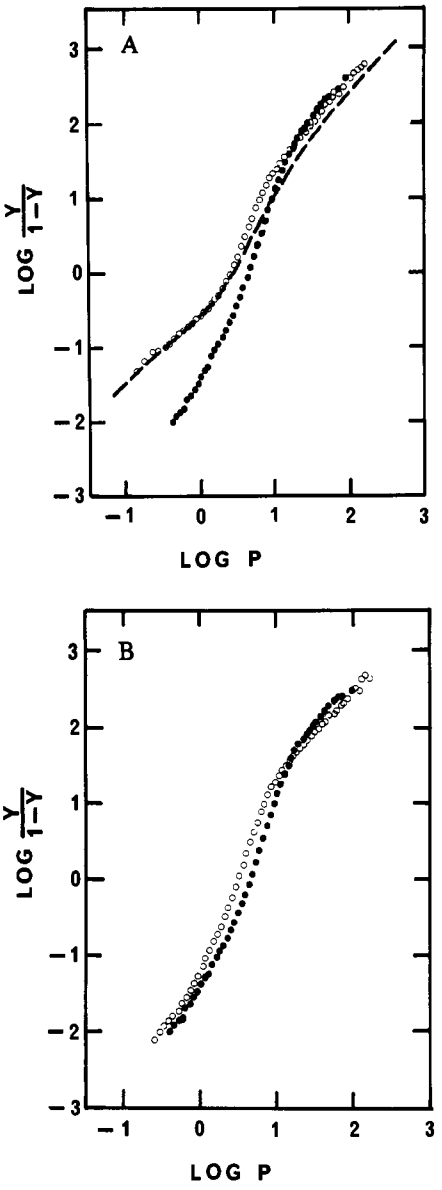

FIGURE 3: Hill plots of oxygen equilibrium curves of XL82β-Hb and Hb A. *Y*, fractional saturation of oxygen; *P*, partial pressure of oxygen in millimeters of mercury (1 mmHg = 133.3 Pa). (A) ○, XL82β-Hb purified by the previous method, at pH 7.47; ●, Hb A at pH 7.4; the broken line was calculated from the published data for XL82β-Hb in 0.1 M phosphate buffer, pH 7.41, at 25 °C [from Table I of Miura et al. (1987)]. (B) ○, XL82β-Hb at pH 7.4 purified by the present method; ●, Hb A at pH 7.4. Other conditions are as follows: Hb concentration, 60 μM on a heme basis; temperature, 25 °C; buffer, 0.05 M Bistris-HCl containing 0.1 M Cl^-.

is responsible for the high-oxygen-affinity impurity that shifted the lower portion of the Hill plot to the left. The fraction of the impurity, which can be estimated by extrapolating the slow phase to zero time (broken line in Figure 4), is about 20% of the total cross-linked Hb. The second-order rate constant for the fast component was 11 M^{-1} s^{-1}, whereas that for slow one was 0.5 M^{-1} s^{-1}, comparable to that for deoxyHb A (0.43 M^{-1} s^{-1}). Another 4-PDS kinetic experiment carried out under a CO gas atmosphere showed no significant difference in 4-PDS reactivity between CO-XL82β-Hb and CO-Hb A (result not shown). This observation is consistent with the oxygen equilibrium curves in Figures 3A. Despite the presence of about 20% impurity, the upper portion of the Hill plot for XL82β-Hb is close to that of Hb A. Thus, the properties of fully liganded impurity seem to resemble those of fully liganded Hb A.

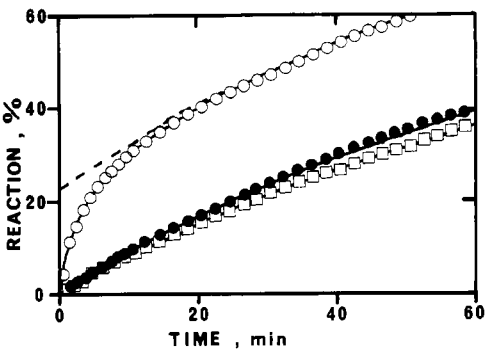

FIGURE 4: Time courses of the reaction of 4-PDS with sulfhydryl groups of deoxyXL82β-Hb and deoxyHb A. ○, XL82β-Hb purified by the previous method; □, XL82β-Hb purified by the present method; ●, Hb A. Conditions were as follows: temperature, 10 °C; buffer, 0.1 M phosphate, pH 7.0; Hb concentration, 50 μM on dimer basis; initial 4-PDS concentration, 480 μM.

It should be mentioned here that this impurity cannot be removed from authentic XL82β-Hb by highly resolving anion-exchange HPLC using a DEAE-5PW column in place of CM52 cellulose column chromatography. We used a gradient elution according to the method of Bucci et al. (1989). After the HPLC purification, there still remained about 20% impurity as judged from its 4-PDS kinetic time course and oxygen equilibrium curve (data not shown).

Further purification described under Experimental Procedures was devised to remove the impurity on the basis of its increased sulfhydryl reactivity under a deoxygenated condition. After one cycle of the purification procedure, 4-PDS kinetic was measured to check the purity of the sample. Although the fraction of the fast component was significantly decreased, nearly 5% impurity still remained. Therefore, we repeated the purification procedure twice[3] in order to diminish the impurity to less than 0.4%. After these steps, purity of XL82β-Hb was confirmed by analytical isoelectric focusing (lanes 2 and 4 in Figure 1) and anion-exchange HPLC using a DEAE-5PW column.

As shown in Figure 2, XL($\alpha\beta^{Cys+}$)($\alpha\beta^{NES}$) seemed to appear as double peaks (peaks B and C). The most reasonable interpretation is that one peak corresponds to XL82β-Hb and the other corresponds to the impurity. This speculation is consistent with the fact that the ratio of peak B to peak D is significantly decreased in the second cycle of purification, and further peak B almost disappeared in the third cycle. Therefore, we assign peak B to the impurity.

The 4-PDS reaction time courses for purified deoxyXL82β-Hb and deoxyHb A, which are superimposable within the limits of experimental error, imply that the cross-linking causes no significant structural perturbation around Cys-93β residues in the deoxy quaternary structure. The leftward shift of the lower portion of the Hill plot for XL82β-Hb purified by the previous method is ascribed to the presence of the impurity and it is never the intrinsic property of pure XL82β-Hb (see Figure 3B).

Identification of Purified XL82β-Hb. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of purified XL82β-Hb revealed two bands of equal intensity, one corresponding to the α monomer and the other to the β dimer (result not shown). Therefore, we used gel-filtration HPLC

[3] We have examined several experimental conditions to increase the efficiency of removal of the impurity without repeating the purification procedure, e.g., increasing the molar ratio of NEM to Hb at the first step of purification. However, such conditions have led a great loss of desired XL82β-Hb, so that we adopted the repetition of the purification method.

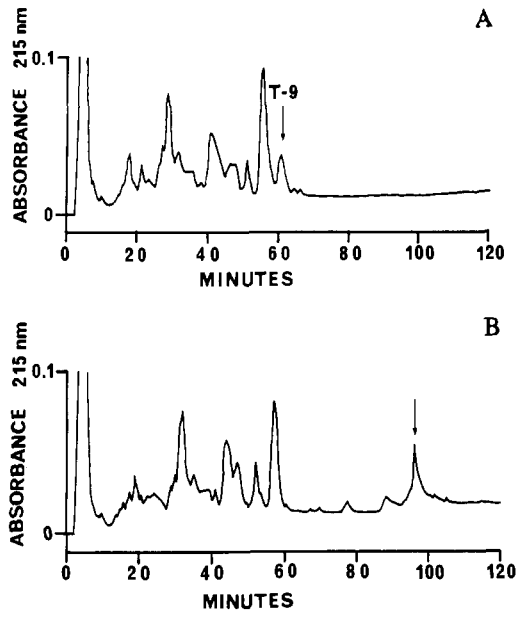

FIGURE 5: Reverse-phase HPLC elution profile of tryptic peptides of the β chains from (A) HbA and (B) XL82β-Hb. Column used as Hi-Pore RP-304. Peptides were eluted by a gradient from 0 to 15% acetonitrile in 10 min and then to 50% acetonitrile in 140 min at a flow rate of 1 mL/min. Arrow in (B) indicates appearance of cross-linked peptide.

in the presence of 0.1% sodium dodecyl sulfate in order to isolate the cross-linked β dimer. Reverse-phase HPLC elution patterns of tryptic digests of native β chain and the cross-linked β dimer are shown in Figure 5. In the case of cross-linked β dimer, the peptide eluted at 61 min was missing and an additional new peak was appeared at 97 min (Figure 5B, arrow). Sequence analysis of these peptides showed that the effluent at 61 min contains T-9 (β67–β82: VLGAFSDGLAHLDNLK) and the new peak at 97 min contains T-9 + T-10 [β67–β95: VLGAFSDGLAHLDNLXGTFATLSELH(C)DK]. We also found that no amino acid is detected at cycle 16 (β82) of T-9 + T-10 from the cross-linked β dimer (we denote this by X), indicative of the presence of a fumaryl bridge between Lys-82β_1 and Lys-82β_2. Thus, we identified the new peak appearing at 97 min as the dimeric peptide (β67–β95)$_2$, which is cross-linked at the position of Lys-82β. These findings strongly indicate that the purified XL82β-Hb sample (peak D in Figure 2) is cross-linked between Lys-82β_1 and Lys-82β_2.

Oxygen Equilibrium Properties of Purified XL82β-Hb. The Hill plots of the oxygen equilibrium curves of purified XL82β-Hb and native Hb A in several solution conditions are shown in Figure 6. The estimated P_{50}, n_{max}, and K_i (i = 1–4) values for those Hbs are listed in Table I. The values of log K_1, log K_4, –log P_{50}, and n_{max} are plotted against pH in Figure 7. The parameter values for XL82β-Hb are similar to those for Hb A in the absence of IHP. Unlike Hb A, the P_{50} value of XL82β-Hb is insensitive to IHP, consistent with the fact that the binding site of DPG (therefore, also IHP) is occupied by the fumaryl group (Walder et al., 1980). In all cases, the n_{max} values of XL82β-Hb are very close to those of Hb A, indicating that the cross-linking between Lys-82β_1 and Lys-82β_2 does not affect the cooperativity of the Hb molecule.

The number of released Bohr protons associated with the first oxygenation step (Δ H_1^+), which is estimated by the formula (German & Wyman, 1937) $\Delta H_1^+ = \Delta \log K_1/\Delta$pH, is about 0.5 at pH 7.4 for XL82β-Hb and Hb A. The total Bohr effect, which is measured by the slope of log P_{50} vs pH plot, is also preserved in XL82β-Hb (Figure 7). Moreoever, the chloride effect is substantially preserved in XL82β-Hb (Table I).

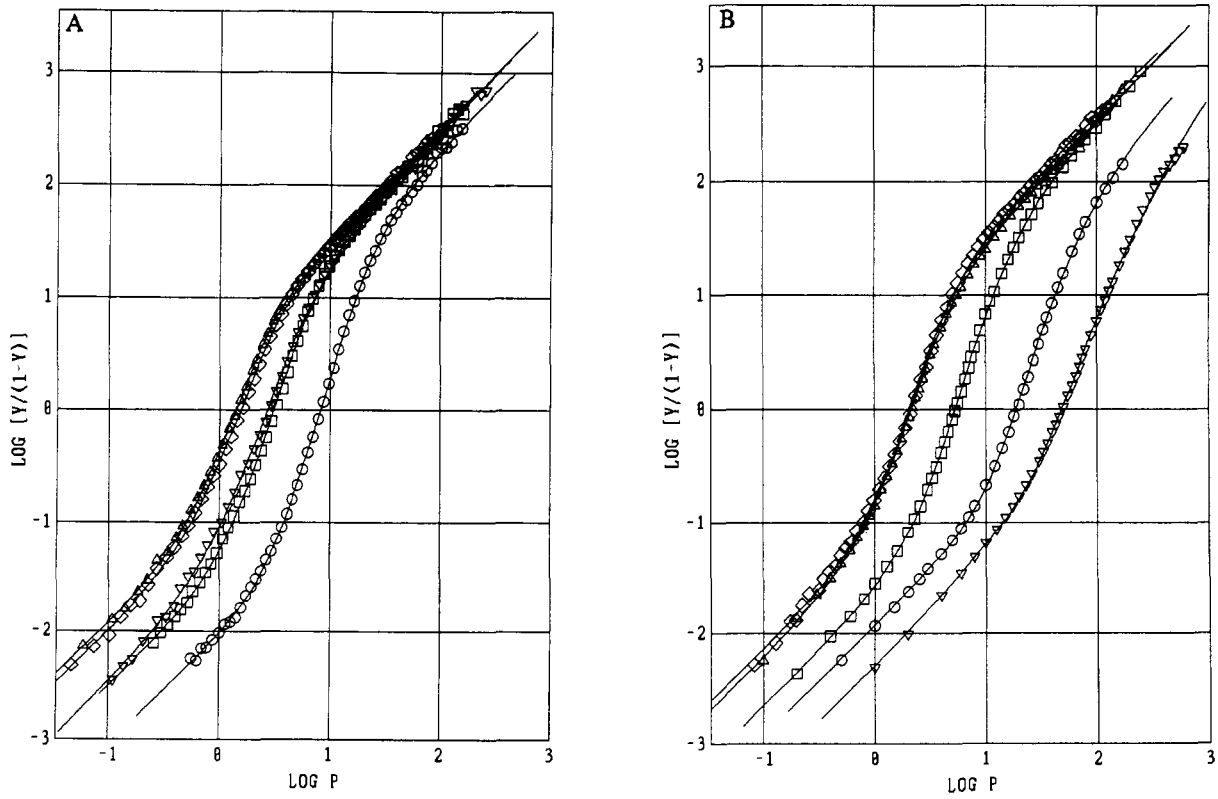

FIGURE 6: Hill plots of oxygen equilibrium curves for (A) XL82β-Hb and (B) Hb A. Y, fractional saturation of oxygen; P, partial pressure of oxygen in millimeters of mercury (1 mmHg = 133.3 Pa). ○, pH 6.4 for XL82β-Hb and pH 6.5 for Hb A; □, pH 7.4; ◇, pH 8.4; △, pH 9.1; ▽, pH 7.4 in the presence of 2 mM IHP. Other conditions are described in Table I. Lines were calculated from the four Adair constants listed in Table I.

Table I: Oxygen Equilibrium Parameters of XL82β-Hb and Hb A

XL82β-Hb								
conditions[a]								
pH	anion	P_{50} (mmHg)	n_{max}	K_1 ($mmHg^{-1}$)	K_2 ($mmHg^{-1}$)	K_3 ($mmHg^{-1}$)	K_4 ($mmHg^{-1}$)	MetHb[b] (%)
6.4	0.1 M Cl^-	8.2	3.28	0.0084	0.023	0.52	2.1	2.4
7.4	0.1 M Cl^-	3.1	2.97	0.030	0.092	1.2	3.0	2.0
8.4	0.1 M Cl^-	1.5	2.53	0.097	0.22	2.4	3.2	1.8
9.1	0.1 M Cl^-	1.4	2.53	0.12	0.15	4.9	3.0	ND[c]
7.4	0.1 M Cl^-, 2 mM IHP	2.7	2.64	0.031	0.37	0.47	3.1	2.2
7.4	7 mM Cl^-	1.7	2.64	0.080	0.17	2.3	3.3	1.7
Hb A[d]								
conditions								
pH	anion	P_{50} (mmHg)	n_{max}	K_1 ($mmHg^{-1}$)	K_2 ($mmHg^{-1}$)	K_3 ($mmHg^{-1}$)	K_4 ($mmHg^{-1}$)	MetHb (%)
6.5	0.1 M Cl^-	18.8	2.88	0.0119	0.011	0.069	1.24	2.0
7.4	0.1 M Cl^-	5.32	3.02	0.0218	0.062	0.30	3.45	2.5
8.4	0.1 M Cl^-	2.09	2.79	0.0720	0.0881	2.31	3.76	3.1
9.1	0.1 M Cl^-	2.15	2.73	0.0595	0.16	1.5	3.33	1.9
7.4	0.1 M Cl^-, 2 mM IHP	48.8	2.53	0.00502	0.013	0.0042	0.915	2.2
7.4	7 mM Cl^-	2.05	2.40	0.0647	0.48	0.45	4.24	3.2

[a] Other conditions were as follows: temperature, 25 °C; Hb concentration, 60 and 600 μM (on a heme basis) for XL82β-Hb and Hb A, respectively; buffer, 0.05 M Tris-HCl or Bistris-HCl. [b] Methemoglobin contents after measurements. [c] Not determined. [d] Data from Imai (1982) except for the data at pH 8.4, which are unpublished.

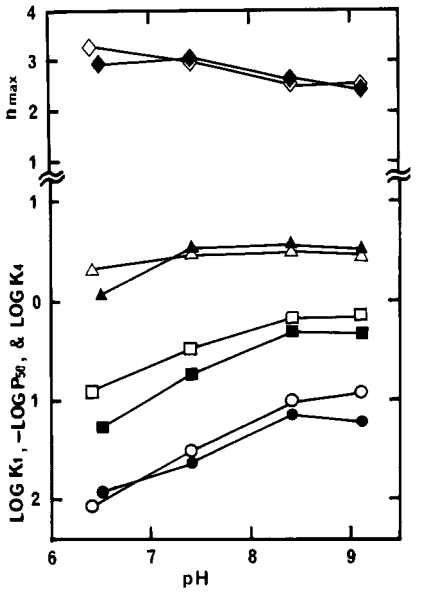

FIGURE 7: pH dependence of oxygen equilibrium parameters of XL82β-Hb (open symbols) and Hb A (closed symbols). The data in Table I are plotted. ○ and ●, log K_1; △ and ▲, log K_4; □ and ■, $-$log P_{50}; ◇ and ◆, n_{max}.

The differences in oxygenation parameter values between our XL82β-Hb and the preparation of Miura et al. (1987) seem to arise from the high-oxygen-affinity impurity in the latter. The major effect of the high-affinity impurity is the increase in the K_1 value: at pH 7.4, the K_1 value, which was observed to be 0.35 $mmHg^{-1}$ (Miura et al., 1987), was found to be 0.030 $mmHg^{-1}$ in our study, showing more than 10-fold lowering. Another striking difference is noted in n_{max} values. In the previous study the n_{max} value of XL82β-Hb at pH 7.4 was 2.3 (Miura et al., 1987), whereas in our study it is as large as 2.97. In conclusion, the cross-linking between Lys-82β_1 and Lys-82β_2 by a fumaryl group affects the oxygenation properties of Hb molecule much less than previously considered.

Comparison with Other Cross-Linked Hbs. Arnone et al. (1977) showed that asymmetric cross-linking between Lys-82β_1 and the α-amino group of Val-1β_2 occurs during the reaction of deoxyHb with NFPLP (2-nor-2-formylpyridoxal 5′-phosphate). An oxygen equilibrium study on NFPLP-cross-linked Hb showed reduced cooperativity (n = 2.2) and about 10-fold lower oxygen affinity compared to unmodified Hb (Benesch et al., 1975). These functional properties have been interpreted by large distortions caused by cross-linking in the oxy quaternary structure. During the normal structural transition of the Hb molecule, the relative distance between Lys-82β_1 and the N-terminus of the β_2 chain is lengthened from 11 Å in the deoxy quaternary structure (Fermi, 1975) to 16 Å in the oxy quaternary structure (Perutz et al., 1968). Thus, the 7.5-Å NFPLP cross-link bridge may destabilize the oxy quaternary structure of Hb molecule.

Recently, Bucci et al. (1989) reported that the reaction of oxy Hb A with mono(3,5-dibromosalicyl) fumarate produces a derivative specifically acylated at the two Lys-82β residues. Upon acylation, free carboxyl groups of the fumaryl residues are introduced inside the β cleft of the Hb molecule, and the introduction of additional electrostatic interactions inside the β cleft results in a pseudo-cross-link, which confers stability to the tetrameric forms of Hb. The oxygen affinity of the pseudo-cross-linked Hb is higher than that of Hb A between pH 6.5 and 7.2. Above pH 7.2 its oxygen affinity is lower than that of Hb A. At all these pH values the cooperativity is lowered, with a Hill coefficient near 2. One may expect that the noncovalent pseudo-cross-link affects the cooperative oxygenation of Hb less extensively than the covalent cross-link reported in the present study. However, such is not the case. The most probable interpretation for the large perturbations caused by the noncovalent pseudo-cross-link is that the two fumaryl groups introduced inside the β cleft are too bulky to maintain the normal allosteric transition of Hb.

Oxygen equilibrium properties have also been reported for other cross-linked Hbs in which the cross-links are not located on the Lys-82β residues, such as Hb cross-linked between the α chains by bis(3,5-dibromosalicyl) fumarate (Chatterjee et al., 1986; Snyder et al., 1987; Vandegriff et al., 1989), carboxymethylated cross-linked Hb (Fantl et al., 1987), and 4,4′-diisothiocyanatostilbene-2,2′-disulfonate- (DIDS-) cross-linked Hb (Kavanaugh et al., 1988). In all cases, these cross-linked Hbs show considerably lower affinity for oxygen and somewhat reduced cooperativity compared to unmodified Hb.

The most recent studies on cross-linked Hbs aim to develop a cell-free blood substitute for clinical use. For such a purpose, oxygen affinity as low as that of whole blood and a stabilized tetrameric nature are the properties required. On the other hand, our aim is to characterize asymmetrically liganded in-

termediate species in the course of oxygenation. The cross-linking between Lys-82β_1 and Lys-82β_2 by the fumaryl group is an excellent chemical modification for our aim.

Nature of Impurity. As described above, we have succeeded in removal of the impurity (or impurities), which shows striking similarities in electrophoretic features to authentic XL82β-Hb. Here, we present several additional results that help us to speculate on the mechanism of impurity formation.

(i) The molar ratio of the cross-linking agent to Hb was decreased from 1:1 to 0.3:1, in order to examine whether or not the fraction of impurity was decreased. However, the fraction of impurity was unchanged.

(ii) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of the impurity-rich component, i.e., peak A in Figure 2, showed both the α monomer and the β dimer bands with nearly equal intensity.

(iii) Tryptic peptide mapping of the β dimer derived from the impurity-rich component revealed differences in peptide patterns from native β chain or the β dimer cross-linked between Lys-82β_1 and Lys-82β_2: T-9 for the impurity-rich component appeared with about half-intensity compared with that of native β chain.

(iv) Oxygen equilibrium curves of the cross-linked Hb preparation, which was contaminated with about 20% impurity, were little affected by addition of IHP.

From (i), it seems unlikely that the impurity is formed by multiple modifications, because, if multiple modifications occurred, the fraction of the impurity should have been decreased under the condition examined here. Moreover, (ii) and (iv) indicate that the impurity contains a covalent cross-link between the two β chains and the cross-link bridge is located at the DPG binding site. Our preliminary mapping data of (iii) suggest that in the impurity one end of fumarate is linked to the Lys-82β_1 residue, whereas the other end is linked to some unknown amino group within the β_2 chain to form an asymmetric intersubunit bridge. This speculation is not inconsistent with other data, although we have not succeeded in finding such an asymmetrically cross-linked peptide. Inspection of the X-ray crystallographic structure of Hb (Perutz et al., 1968; Fermi, 1975) shows that the α-amino group of Val-1β or the ϵ-amino group of Lys-144β is a possible candidate for the unknown amino group.

In fact, the cross-linking between Lys-82β_1 and the α-amino group of Val-1β_2 occurs in the reaction of deoxyHb with NFPLP, although the length of this cross-link bridge, i.e., 7.5 Å, is slightly longer than that of the fumaryl bridge, i.e., 6.8 Å (Arnone et al., 1977). In this cross-linked derivative the N-terminus of the β_2 chain moves about 3 Å toward Lys-82β_1 in order to complete the cross-linking. This observation supports the stereochemical possibility of the cross-linking between Lys-82β_1 and N-terminal of the β_2 chain by the fumaryl group. However, it is known that the α-amino group of Val-1β shows its pK value of about 7 regardless of the quaternary structure (Garner et al., 1975). Thus, it is rather unlikely that this type of cross-linking, even if it occurred, could not be distinguished from normal cross-linking by anion-exchange HPLC at pH 8. Moreover, as discussed before, an oxygen equilibrium study on NFPLP-cross-linked Hb showed very low affinity for oxygen (Benesch et al., 1975), in striking contrast to the high oxygen affinity of the impurity in our study. In the oxy quaternary structure, Lys-82β_1 and the N-terminal of the β_2 chain are too far apart (about 16 Å) (Perutz et al., 1968) to be connected by NFPLP (7.5 Å) or fumarate (6.8 Å), so that cross-linking in this way is expected to stabilize the low-affinity conformation of Hb. On the other hand, the distance between Lys-82β_1 and Lys-144β_2 is shortened upon oxygen binding. Thus, cross-linking between Lys-82β_1 and Lys144β_2 remains a probable candidate for the chemical modification pertaining to the high-oxygen-affinity impurity.

Conclusions. Oxygen equilibrium properties of highly purified XL82β-Hb are very similar to those of native Hb A, indicating that the cross-linking between Lys-82β_1 and Lys-82β_2 by a fumaryl group little affects the cooperative oxygenation of Hb molecule. This finding is of great significance for future studies on intermediately liganded states of Hb with cross-linked asymmetrically modified hybrid Hbs. Another significant finding is the discovery of the electrophoretically silent impurity, which shows much higher oxygen affinity than XL82β-Hb. Most previous reports on XL82β-Hb and cross-linked asymmetrically modified hybrid Hbs should be subject to the criticism of sample contamination.

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Synthesis and Evaluation of an Inhibitor of Carboxypeptidase A with a K_i Value in the Femtomolar Range†

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ABSTRACT: Comparative studies among a series of tripeptide phosphonate inhibitors of the zinc peptidase carboxypeptidase A indicate that incorporation of the phosphonic acid analogue of valine at the P_1 position results in significantly higher affinity than the glycine, alanine, or phenylalanine analogues. When applied to the tripeptide analogue Cbz-Phe-Val^P-(O)Phe [ZFV^P(O)F], determination of the inhibition constant K_i was complicated by the very slow rate of dissociation. The rate of exchange of [^{3}H]ZFV^P(O)F with enzyme-bound [^{14}C]ZFV^P(O)F was followed for periods of 3–4 months to measure dissociation rate constants in the range of (1.7–4.4) × 10^{-9} s^{-1}, corresponding to half-lives of 5–13 years. Although the on- and off-rate constants differ for different carboxypeptidase isozymes, their ratios, corresponding to the inhibition constants K_i, are consistently in the range of 10–27 fM. Both the inhibition constants and the dissociation rate constants appear to be the lowest values yet determined for an enzyme–small inhibitor interaction.

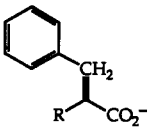
Considerable attention has been devoted to the synthesis of enzyme inhibitors for better understanding of enzyme mechanism, to probe the forces that bind small molecules to proteins, and for the purpose of drug design. The effectiveness of a molecule as a reversible enzyme inhibitor is gauged quantitatively, usually by the inhibition constant K_i but also by the rate constant for dissociation, k_{off} (Segel, 1975; Morrison & Walsh, 1987; Schloss, 1988a):

$$E + I \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E{\cdot}I \qquad K_i = k_{off}/k_{on} = [E{\cdot}I]/[E][I] \quad (1)$$

As the strategies for inhibitor design are refined, the operational definitions of "potent inhibition" and "slow dissociation" change. In this respect, the evolution of carboxypeptidase A (CPA)[1] inhibitors is representative, as the partial list in Table I indicates. Although the progression of designs has not led monotonically to enhanced affinity, it is interesting to note that virtually every inhibitor in Table I was characterized as "potent" at the time it was reported.

As one of the prototypic zinc proteases, CPA occupies a prominent position in enzymology. It has been the subject of considerable scrutiny and ample debate in attempts to correlate structural information with mechanistic insights [e.g., Christianson and Lipscomb (1989)], and it has served as a model in the development of inhibition strategies that can be

Table I: Advancement in Inhibitor Design: Carboxypeptidase A



inhibitor (R)	K_i (nM)	reference
H	6200	Elkins-Kaufman and Neurath (1949)
$^-O_2CH_2$	450	Byers and Wolfenden (1973)
$^{2-}O_3PNH$	5000	Kam et al. (1979)
$HSCH_2$	11	Ondetti et al. (1979)
$^{2-}O_3PO$	140	Hofmann and Rottenberg (1980)
Cbz-Gly(PO_2^--NH)[a]	90	Jacobsen and Bartlett (1981)
O=$CHCH_2$	<480	Galardy and Kortylewicz (1984)
$^{2-}O_3PCH_2$	220	Grobelny et al. (1985)
$CF_3C(OH)_2CH_2$	200	Gelb et al. (1985)
Cbz-Phe-Ala-(PO_2^--O)[b]	0.001	Hanson et al. (1989)

[a] = ZG^{P}F. [b] = ZFA^P(O)F

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[1] Abbreviations: CPA, carboxypeptidase A; ZFV^P(O)F, the phosphonate ester analogue of Cbz-Phe-Val-Phe in which the Val-Phe peptide linkage has been replaced with PO_2^--O (related inhibitors are abbreviated similarly by using the single-letter amino acid code); dpm, decompositions per minute; SA, specific activity; LSC; liquid scintillation counting; fuaFF, furanacryloyl-L-phenylalanyl-L-phenylalanine.

0006-2960/91/0430-8165$02.50/0