The Preparation and Properties of the Isolated α and β Subunits of Hemoglobin A*

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ABSTRACT: Human adult hemoglobin has been separated into the constituent α and β chains by a modification of the method of Bucci and Fronticelli [Bucci, E., and Fronticelli, C. (1965), *J. Biol. Chem. 240*, PC551]. Methods are described for the complete removal of mercury from both chains. The purity of the isolated subunits was established by direct analysis for mercury, SH group determination, starch gel electrophoresis, spectral analysis, and ultracentrifugation. The susceptibility to oxidation of the two kinds of subunits was found to differ significantly. Reconstitution of hemoglobin A from the isolated chains led to oxygen dissociation curves which were indistinguishable from those of the original protein.

Since a study of the isolated α and β subunits of hemoglobin is obviously highly desirable for an understanding of the molecular properties of the parent molecule, methods which can yield these subunits in a "native" state are of the greatest interest. We have reinvestigated the procedure of Bucci and Fronticelli (1965) and introduced some crucial modifications which make it possible to prepare α and β chains that can be recombined to give hemoglobin indistinguishable from the starting material as judged by a number of criteria.

The Bucci method consists essentially of three separate steps: (a) dissociation of the hemoglobin molecule by treatment with *p*-mercuribenzoate in slightly acid solution; (b) separation of the mercurated chains by means of a pH gradient on a carboxymethylcellulose column; (c) conversion of the isolated mercury derivatives of the α and β chains into the SH form by dialysis against thiols.

This procedure leads to a very satisfactory separation of the constituent chains of hemoglobin in the form of their mercury derivatives. The method of mercury removal described by Bucci and Fronticelli (1965) is, however, not satisfactory, since it is incomplete, particularly in the case of the β chains. The low SH titers reported by these authors indicate the difficulty of removing mercury completely.

Experimental Section

Materials

Human hemoglobin was prepared as described previously (Benesch and Benesch, 1962a) from the blood of two young, healthy, adult males. Separation into the constituent chains was begun within 24–48 hr after collection of the blood. The source of hemoglobin H for these experiments was a 17-year-old white female. Hemoglobin concentration was determined spectrophotometrically at 540 mμ after conversion to methemoglobin cyanide (ϵ 11.5 $\times$ 10^3/heme).

Carboxymethylcellulose was purchased from Bio-Rad Laboratories; Sephadex G-10 and G-25 were manufactured by Pharmacia, Uppsala, Sweden. 2-Aminoethyl hydrogen sulfate was obtained from Aldrich and recrystallized from hot water. The source of *N*-acetyl-DL-homocysteine thiolactone and glutathione was Schwarz Bioresearch, that of 98% thioglycolic acid Evans Chemetics. Calbiochem supplied dithiothreitol and mercaptoethanol; diphenylthiocarbazone (dithizone) and thiourea were bought from Fisher. Sodium *p*-hydroxymercuribenzoate was a Sigma product. Gel electrophoresis was carried out with Connaught hydrolyzed starch. All other chemicals used were reagent grade and were purchased from standard sources.

Preparative Methods

Isolation of Mercurated α and β Chains. All separations were carried out with oxyhemoglobin at 5°. In general the procedure followed was identical with that described by Bucci and Fronticelli (1965). It was noted, however, that the stability of the separated chains could be significantly increased by adding sufficient phosphate buffer, pH 7.5, to the eluted samples to make the final concentration 0.1 M. This is particularly advantageous in the case of the β chains, which are eluted at a more acid pH. The separated mercury

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derivatives of the α and β chains were then concentrated by ultrafiltration through collodion membranes with a porosity less than 5 mμ (Schleicher and Schuell, Keene, N. H.).

Removal of Mercury. A. DIALYSIS AGAINST THIOLS. Dialysis for 12 hr against 0.05% thioglycolate in 0.01 M phosphate buffer, pH 7.4, containing 5×10^{-4} M Versene, followed by dialysis for 30 hr against several changes of the buffer was recommended by the Rome group for this purpose (Bucci and Fronticelli, 1965; Antonini *et al.*, 1965). When this procedure was followed, the α and β chains were found to contain 0.025 and more than 0.35 atom of mercury/chain, respectively; *i.e.*, mercury removal was 97.5% complete in the case of the α and less than 85% complete in the case of the β chains. In addition, both chains showed changes in the spectrum, corresponding to about 13% methemoglobin. Such deterioration, undoubtedly caused by coupled oxidation, occurred even more rapidly when α chains were dialyzed against other thiols, *e.g.*, mercaptoethanol or dithiothreitol (Cleland, 1964). These difficulties led us to the use of separate methods for the removal of mercury from the two kinds of chains.

B. DEMERCURATION OF α CHAINS. Since the α chains are so very unstable in the presence of free thiols, we resorted to the use of thiolated Sephadex. In this material SH groups are linked covalently to the polysaccharide matrix and are remarkably stable to air oxidation, presumably as a result of steric restraint (Jellum, 1964). The preparation of this polymer was first described by Eldjarn and Jellum (1963) based on the thiolation reactions of Benesch and Benesch (1958, 1959). The Sephadex is first aminated with 2-aminoethyl hydrogen sulfate and the resulting NH_2-Sephadex is thiolated with *N*-acetylhomocysteine thiolactone to yield SH Sephadex.

The amination was carried out on Sephadex G-25 (coarse) exactly as described by Eldjarn and Jellum (1963). The thiolation procedure was modified in several respects.

NH_2 Sephadex (corresponding to 32 g of original Sephadex) was suspended in 600 ml of water. *N*-Acetylhomocysteine thiolactone (6 g) was added and the solution protected from light with aluminum foil. $AgNO_3$ (1 N) and 1 N NaOH were added from two burets with vigorous magnetic stirring in small increments, so as to maintain the pH between 8.5 and 9, until 38 ml of $AgNO_3$ and 46–50 ml of NaOH had been added (about 2 hr). A substantially higher degree of thiolation was achieved by maintaining the pH between 8.5 and 9 rather than at 7.5, although care must be taken not to exceed pH 9 to avoid reduction of silver ions.

The clear yellow supernatant solution was siphoned off and replaced by water. Solid thiourea (50 g) was then added followed by sufficient 1 N HNO_3 to bring the pH to 2. The SH Sephadex was washed with 1 N thiourea in 0.01 N HNO_3 until it was free of silver. (The suspension remained colorless after addition of alkali and passage of H_2S.) Subsequent washing with water as recommended by Eldjarn and Jellum (1963) is ineffective in removing excess *N*-acetylhomocysteine thiolactone and *N*-acetylhomocysteine. As a result, erroneously high SH titers for the final product are obtained and a considerable proportion of the SH groups will be present as noncovalently bound thiol. This can be avoided by washing with 0.1 M phosphate buffer, pH 7.5, instead of water. Even then it is remarkably difficult to remove the thiolactone completely, several days of column washing being necessary to reduce the absorbance of the effluent at 236 mμ to neglegible values.

The purified SH Sephadex was sampled by pouring a suspension into 1-ml tuberculin syringes with no. 20 needles and allowing it to settle to a given height with a flow of buffer as in a column. The known volume (0.2–0.3 ml) was transferred quantitatively to a flask and shaken for 1 hr with excess *p*-mercuribenzoate. The free *p*-mercuribenzoate was titrated spectrophotometrically with glutathione (Boyer, 1954) after removal of the Sephadex by filtration. In this way different batches of SH Sephadex were found to contain from 7 to 10 μmoles of SH/ml of Sephadex column. Therefore a column of 150-ml of SH Sephadex (30 cm in height) provided a 1000-fold excess of SH over mercury when 1 ml of a 2% solution of α chains was applied. Phosphate buffer (0.1 M, pH 7.5) was then passed through the columns at 5° at a rate which required 5–6 hr for elution of the α chains. After use the columns were regenerated with 20 ml of 0.1 M mercaptoethanol and washed with the buffer until the nitroprusside test was negative.

C. DEMERCURATION OF β CHAINS. As pointed out above, it is much more difficult to remove mercury quantitatively from β chains than from α chains. Thus it was found impossible to remove all the mercury from β chains on thiolated Sephadex columns. Not surprisingly, thiolated Sephadex G-50 was more effective than G-25, but it proved to be too cumbersome for routine use.

Since β chains proved to be more stable than α chains to oxidation in the presence of free thiols, it was possible to adopt the following procedure. Columns of Sephadex G-10, 30 cm in height and 150 ml in volume, were equilibrated with 0.1 M phosphate buffer, pH 7.5. A 0.1 M aqueous solution of mercaptoethanol (20 ml) was applied to the top of the column. After all the mercaptoethanol had entered the column, 2 ml of a 2% solution of mercurated β chains was layered on the column and passed through it with a flow of phosphate buffer in the course of 5–6 hr. On the average, 20 ml of buffer passed after the complete elution of the β chains and before the first appearance of mercaptoethanol.

Reconstitution of Hemoglobin A. Reconstitution was performed by mixing equimolar amounts of the oxy forms of α and β chains at a total hemoglobin concentration of 1–1.5% in 0.1 M phosphate buffer, pH 7.5. Since, in agreement with previous workers (Huehns *et al.*, 1964; Ranney *et al.*, 1965), we found the recombination of α and β chains not to be instantaneous,

the solutions were left at room temperature for 1 hr.

Isolation of Hemoglobin H. Hemoglobin was prepared from the patient's blood by the usual method (Benesch and Benesch, 1962a). It was then dialyzed for 4 hr against two changes of 2 l. of 0.01 M phosphate buffer, pH 6.7. The 9% hemoglobin solution (16 ml) was applied to a 26-cm carboxymethylcellulose column equilibrated with 0.01 M phosphate buffer, pH 5.9. The hemoglobins were eluted with the same pH gradient as that used for the separation of mercurated α and β chains. The elution diagram is shown in Figure 1.

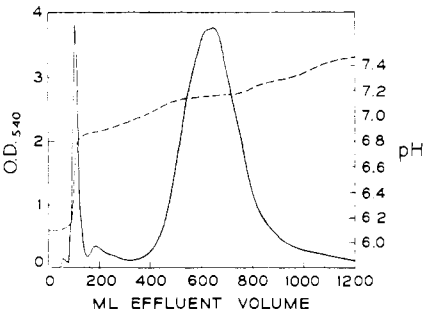

FIGURE 1: Elution diagram of hemolysate containing hemoglobin H. The solid line represents the optical density at 540 mμ and the broken line the pH of the eluate. The elution rate was 40 ml/hr. Hemoglobin H was collected between 100 and 130 ml. Hemoglobin A, which amounted to about 90% of the total hemoglobin, was eluted between 400 and 900 ml.

Analytical Methods

Mercury Analysis. Since complete absence of mercury in the isolated chains was regarded as very important and determination of the number of SH groups in the protein is not an adequate criterion for establishing this, direct mercury assays were performed. The method used was based on the work of Jacobs *et al.* (1960) and Jacobs and Singerman (1962).

Samples (1 ml) containing 1–2% hemoglobin were treated with 2 ml of concentrated H_2SO_4 in 125-ml glass-stoppered erlenmeyer flasks placed in an ice bath. $KMnO_4$ (10 ml of 6%) was then added and the mixture warmed on a hot plate to about 70°. After standing for 15 min at room temperature the warming and standing were repeated twice more. The sample was then decolorized by the dropwise addition of 20% hydroxylamine hydrochloride (purified by extraction with dithizone) and 20 ml of water was added.

The samples were transferred quantitatively to Mojonnier tubes (Jacobs and Singerman, 1962), 20 ml of 0.001% dithizone in carbon tetrachloride was added, and the tubes were shaken vigorously for 1 min. CCl_4 (2.5 ml) was then added to bring the organic layer to the top of the constriction. The aqueous layer was discarded and the organic layer extracted three times with 25-ml portions of 7 N ammonia. It was then filtered through Whatman No. 42 filter paper. The solution and washings were made up to volume in a cylindrical cuvet of 10-cm light path and 32-ml capacity and the optical density was read at 490 mμ. Direct light must be excluded after the addition of dithizone. Under these conditions the absorbance due to 0.01 μatom of mercury was 0.18. Therefore, amounts of mercury as low as 0.002 μatom could be detected. This corresponds to about 0.2% of the mercury originally bound with the amounts of hemoglobin used for analysis.

Determination of SH Groups. Sulfhydryl groups were titrated spectrophotometrically with *p*-mercuribenzoate at pH 7.0 as described previously (Boyer, 1954, Benesch and Benesch, 1962b).

Oxygen Equilibrium Curves. These data were obtained using the recently described tonometers of Benesch *et al.* (1965). Measurements were made in 0.1 M phosphate buffer with 0.3% hemoglobin solution at 30°. This temperature was chosen because of the high oxygen affinity of the isolated chains. The results for α and β chains were calculated from eq 1 and 2 (Benesch *et al.*, 1965) rather than eq 3–5 because of the slight differences in the spectra of the isolated chains as discussed below. Corrections for bound oxygen were applied when necessary.

Molecular Weight Determinations. These were determined at 5° in the Spinco Model E analytical ultracentrifuge by the "Archibald method" as described by Schachman (1957). All other details were identical with those described previously (Benesch *et al.*, 1964a, 1962b).

Starch Gel Electrophoresis. Horizontal electropherograms were obtained by the method of Smithies (1955) using the continuous buffer system of Smithies (quoted in Bucci *et al.*, 1965b).

Results and Discussion

The results of SH determinations and mercury analyses of several preparations of α and β chains are shown in Table I. It is clear that the proteins were free of mercury and that their SH titers were theoretical within the experimental error. In addition, the absence of small molecule thiols was ascertained by testing an ultrafiltrate of each sample by the prussian

TABLE I: Mercury and SH Analyses of Subunits.

Chain	No. of Prepn	Hg (atom/chain)	SH (residues/chain)
α	6	0.002 ± 0.001	1.03 ± 0.03
β	6	0.003 ± 0.001	2.01 ± 0.02
$\alpha + \beta$	1		0.62
Hb A	3		0.62 ± 0.03

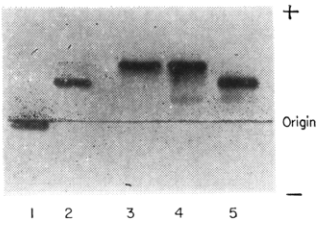

FIGURE 2: Starch gel electrophoresis of hemoglobin and subunits. 1, α chains; 2, $\alpha + \beta$ recombined; 3, β chains; 4, Hb H; 5, Hb A. Migration time, 2 hr. Stained with Amido Black 10B.

blue and nitroprusside methods. In agreement with our previous finding on Hb H, all the SH groups of the isolated β chains (and also of the α chains) were found to react rapidly with *p*-mercuribenzoate at pH 7.0. It can also be seen that on recombination, the number of SH groups per chain decreases from 1.5 (the arithmetic average for the SH content of α and β chains) to 0.6, in good agreement with the value found for native hemoglobin (Table I).[1] Analysis by the method of Evelyn and Malloy (1938) showed that neither the α nor the β chains contained measurable amounts of methemoglobin.

Oxidation of α and β Chains. Oxidation to the ferric state of both α and β chains leads to very rapid denaturation and precipitation of the protein. On the other hand, it is possible to convert both subunits to stable cyanomet derivatives by oxidation with ferricyanide in the presence of cyanide. It was found that the two kinds of subunits show an interesting difference in their reactivity toward the oxidizing agent. As in the case of hemoglobin A, the conversion of α chains from the Fe^{2+} to the $Fe^{3+}CN$ complex with 1 equiv of ferricyanide and 2 equiv of cyanide at room temperature and pH 7 is quantitative (Table II). However, under the same conditions the hemes of the β chains are only partially oxidized (Table II).

All the ferricyanide is quantitatively accounted for by concomitant oxidation of SH groups (Table II). Oxidation as a function of temperature revealed that the rates of the 2 competing reactions have a markedly different temperature dependence. This made it possible to eliminate oxidation of SH groups and achieve quantitative conversion of Fe^{2+} to $Fe^{3+}CN$ at 37° (Table II). The SH groups of Hb H were likewise found to be unusually susceptible to oxidation (Table II). As expected, β chains containing 2 equiv of *p*-mercuribenzoate/chain were oxidized quantitatively to cyanomethemoglobin by 1 equiv of ferricyanide at any temperature.

Starch Gel Electrophoresis. The electropherograms reproduced in Figure 2 show that both the α and the β chains migrate as single components with the expected relative mobility and that the mobility of the β chains is the same as that of isolated hemoglobin H.[2]

Single bands for isolated β chains were also obtained by Bucci *et al.* (1965a) but, in contrast to our experience, three separate α-chain bands were observed by these workers. This difference cannot be accounted for by the buffer systems used, since even in the discontinuous buffer system used by Bucci *et al.* (1965a), our α chains migrated as a single component.

Reconstitution led to the $\alpha + \beta$ zone in Figure 2 with a mobility identical with that of hemoglobin A. As can be seen, a faint band with the mobility of β chains is also present.

Molecular Weights. In agreement with our previous results on hemoglobin H (Benesch *et al.*, 1961, 1962a) and with those of Bucci *et al.* (1965a) on isolated β chains we found the β chains to be tetrameric since the weight-average molecular weight for a 1% solution was 67,000. It was already shown by Bucci *et al.* (1965a) and by Ranney *et al.* (1965) that the state of aggregation of α chains varies with conditions, primarily with concentration. The values for the weight-average molecular weight of our preparation

TABLE II: Oxidation of Hemoglobin Derivatives.[a]

Hemoglobin	Temp (°C)	Equiv/16,500 g $Fe^{2+}O_2$[b]	$Fe^{3+}CN$[b]	SH Found[c]	SH Calcd[d]
Hb A	25	0.02	0.98	...	...
α-SH	25	0.03	0.97	...	...
β-SH	0	0.43	0.57	1.53	1.57
β-SH	25	0.21	0.79	1.82	1.79
β-SH	37	0.04	0.96	1.98	1.96
Hb H	25	0.16	0.84	...	...
Hb H	37	0.04	0.96	...	...
β-Hg	25	0.03	0.97	...	...

[a] Reaction mixture: hemoglobin, 1.8×10^{-4} M (heme basis); $K_3Fe(CN)_6$, 1.8×10^{-4} M; NaCN, 3.6×10^{-4} M; phosphate buffer, pH 7.0, 0.1 M. Reaction time: 1 hr. [b] Calculated from the absorbance at 540, 560, and 576 mμ on the assumption that $Fe^{2+}O_2$ and $Fe^{3+}CN$ are the only hemoglobin derivatives present. [c] These values were kindly determined by Dr. H. Sakai by his very sensitive method for protein SH groups (Sakai, 1960), since the solutions were too dilute for accurate spectrophotometric titration with *p*-mercuribenzoate. [d] Calculated by subtracting the corresponding value for $Fe^{2+}O_2$ from 2.00, the SH content of untreated β chains.

[1] Human hemoglobin A contains six SH groups/mole, of which only the two β93 SH groups react with *p*-mercuribenzoate under the conditions used. Therefore the expected titer per chain would be 0.5.

[2] It will be noted that a small amount of a slower component, with the mobility of hemoglobin A_2, is present in the hemoglobin H.

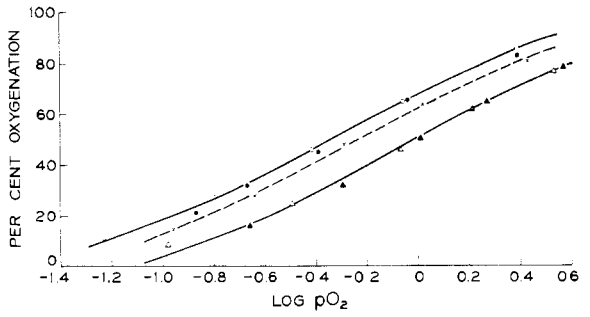

FIGURE 3: Oxygen equilibrium curves of isolated α chains, β chains, and hemoglobin H. O—O, β chains, pH 7.02; ●—●, β chains, pH 7.53; x- - - -x, Hb H, pH 7.02; △—△, α chains, pH 7.02; ▲—▲, α chains, pH 7.53. Hemoglobin concentration 0.3% in 0.1 M phosphate buffer, temperature 30°.

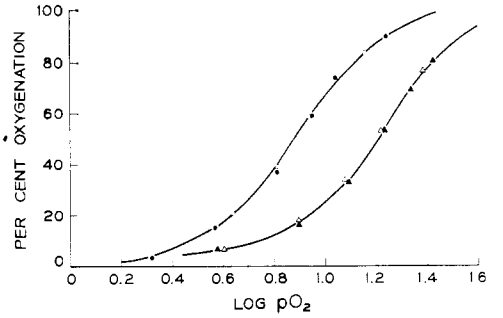

FIGURE 4: Oxygen equilibrium curves of native and reconstituted hemoglobin A. ●—●, native Hb A, pH 7.53; ▲—▲, native Hb A, pH 7.02; O—O, $\alpha + \beta$ recombined, pH 7.53; △—△, $\alpha + \beta$ recombined, pH 7.02. Hemoglobin concentration 0.3% in 0.1 M phosphate buffer, temperature 30°.

of α chains ranged from 23,000 to 30,000. The influence of concentration was evident from the decrease of the molecular weight measured at the meniscus with both the speed and the time of centrifugation. In sedimentation velocity experiments both the α and the β chains migrated as single peaks.

Absorption Spectra. As reported previously, the absorption spectra of the subunits are, in general, quite similar to those of the parent molecule (Antonini *et al.*, 1965). Among the differences the most striking is the broader and flatter Soret band of the deoxy forms of both subunits, originally observed with Hb H (Benesch *et al.*, 1964b).

As expected from the tryptophan content, the absorption of isolated β chains and hemoglobin H in the region of 270–280 mμ is somewhat higher than that of hemoglobin A, whereas that of the isolated α chains is lower. The position of the absorption maximum of the β chains and of hemoglobin H is very close to that of hemoglobin A, *i.e.*, 275–276 mμ The absorption maximum of the isolated α chains. on the other hand is displaced significantly toward shorter wavelengths, *i.e.*, 272 mμ.

Although the visible spectra of isolated α and β chains are very similar to that of hemoglobin A, a definite displacement of the absorption maximum at 576 mμ by about 2 mμ toward the red is observed in the case of β chains and hemoglobin H. This is not so for the α chains. For this reason the oxygen saturations were calculated by a method which was independent of the spectral differences as stated in the experimental part.

Oxygen Equilibria. The oxygen equilibrium curves of the isolated subunits (Figure 3) clearly bear out our original result on Hb H, *i.e.*, the absence of allosteric effects (Benesch *et al.*, 1961). Thus neither the α nor the β chains show any Bohr effect or heme–heme interaction (Table III). The oxygen affinity of the isolated chains is 10–20 times higher than that of the parent hemoglobin. This is in qualitative agreement with the finding of Antonini *et al.* (1965) and of Ranney *et al.* (1965).

TABLE III: Oxygenation Parameters of Hemoglobin[a] and Its Subunits.

	Log $p_{1/2}$[b] pH 7.02	Log $p_{1/2}$[b] pH 7.53	n[c]	$\Delta \log p_{1/2}$ / ΔpH
α chains	0	+0.01	1.0	0
β chains	−0.34	−0.32	1.0	0
Hb H	−0.24	...	1.0	...
$\alpha + \beta$ recombined	+1.22	+0.88	2.7	−0.67
Hb A	+1.22	+0.88	2.7	−0.67

[a] Hemoglobin concentration 0.3% in 0.1 M phosphate buffer; temperature 30°. [b] $p_{1/2}$ is the oxygen pressure at one-half saturation. [c] n is the exponent in the Hill equation: $(HbO_2)/(Hb) = K_p{}^n$.

It is highly significant that the oxygen-binding curve of naturally occurring β chains, *i.e.*, Hb H, differs only so slightly from that of the α chains isolated from Hb A (Figure 3). The α chains have a significantly lower affinity for oxygen than the β chains, as was already shown by Ranney *et al.* (1965) in comparing Hb H with α chains isolated by a different method.

The most gratifying result of this investigation is the complete coincidence of the oxygen equilibrium curves of Hb A reconstituted from isolated chains with those of the original protein (Figure 4). It should be emphasized that this can be achieved only with completely mercury-free chains, since even in the presence of small amounts of mercury, reconstitution led to oxygen dissociation curves which were flatter and displaced to the left. This is reflected by the satis-

factory curves of Ranney *et al.* (1965) who reconstituted their hemoglobin from hemoglobin H and α chains prepared without mercury. By contrast the low n values reported by Antonini *et al.* (1965) for their reconstituted hemoglobin curves as well as the low SH titers of their β chains make it highly probable that mercury was present.

The results of the present investigations therefore again bear out the principle that two kinds of chains are necessary for a normally functioning hemoglobin. Furthermore as long as the subunits are in a "native" state, they recombine quickly and quantitatively to form the original native molecule.

Acknowledgments

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