Interaction between Cytochrome b_5 and Human Methemoglobin†

Marcia R. Mauk* and A. Grant Mauk*

ABSTRACT: Complex formation between purified human methemoglobin and the tryptic fragment of bovine liver cytochrome b_5 has been demonstrated by the observation of a difference spectrum produced on mixing the two proteins. The intensity of this difference spectrum has been used to determine the stoichiometry of the complex formed and its stability under a variety of conditions. At pH 6.2 [25 °C (cacodylate buffer), μ = 2 mM], the complex has a stoichiometry of 1:1 (heme:heme) with a stability constant, K_A, of $(3 \pm 2) \times 10^5$ M^{-1}. This stability constant is dependent on ionic strength, decreasing to a value of $(9 \pm 3) \times 10^3$ M^{-1} at μ = 12 mM [pH 6.2 (cacodylate buffer), 25 °C]. Analysis of this dependence by fitting the data to a form of the Debye–Hückel equation produces a charge product of -64 ± 14 which is in reasonable agreement with the value anticipated on the basis of the amino acid sequences of the two proteins. Determination of the pH dependence of K_A revealed that the complex is most stable at slightly acidic pH (pH 6.0–6.2) or, in other words, at a pH that is approximately midway between the isoelectric pH values of cytochrome b_5 and methemoglobin. The variation of K_A with temperature is consistent with $\Delta H° = -10 \pm 3$ kcal/mol and $\Delta S° = -12 \pm 10$ eu [pH 6.2 (cacodylate buffer), μ = 5 mM]. Together, these results generate a model for cytochrome b_5–methemoglobin interaction in which each hemoglobin subunit binds one cytochrome b_5 by means of complementary charge interactions between oppositely charged groups on the two proteins. The probable sites of cytochrome b_5 binding on hemoglobin are discussed.

In erythrocytes, methemoglobin reduction to deoxyhemoglobin occurs principally through the action of the enzyme NADH:methemoglobin reductase (Gibson, 1948). Hultquist and co-workers have demonstrated that this reaction is mediated by a soluble cytochrome b_5 present in erythrocytes such that methemoglobin reaction involves a direct protein–protein electron transfer from ferrocytochrome b_5 to methemoglobin (Hultquist & Passon, 1971; Sannes & Hultquist, 1978; Hultquist et al., 1981). NADH:methemoglobin reductase, therefore, is more accurately regarded as a cytochrome b_5 reductase related to the enzyme present in liver (Goto-Tamura et al., 1976; Kuma et al., 1976) and other tissues (Kaplan et al., 1974; Leroux et al., 1975; Beauvais et al., 1976).

As the interaction of cytochrome b_5 with methemoglobin remains largely uncharacterized, we have undertaken an analysis of complex formation between these two proteins as a first step toward a better understanding of this important facet of hemoglobin function. The interaction of these two proteins is also of more fundamental significance insofar as it is representative of the general phenomenon of heme protein–heme protein electron transfer. With the three-dimensional structures of both cytochrome b_5 and methemoglobin known to high resolution (Mathews et al., 1979; Ladner et al., 1977), this protein pair provides an attractive model system for a detailed mechanistic characterization of protein–protein electron transfer between proteins that are physiological redox partners.

Experimental Procedures

The tryptic fragment of cytochrome b_5 was purified from fresh beef liver to an $A_{412.5}/A_{280}$ ratio of 6.0 as previously described (Reid & Mauk, 1982). This cytochrome is very similar in structure to human erythrocyte cytochrome b_5 (Imoto, 1977) and is much more readily prepared in the large amounts required for the present studies. Human oxyhemoglobin A_0 was prepared from outdated blood by the method of Williams & Tsay (1973) and stored in liquid nitrogen. Conversion to methemoglobin was achieved by adding a 4-fold molar excess of potassium ferricyanide (per heme) and allowing the reaction to proceed for 1 h at room temperature. Excess ferricyanide and ferrocyanide produced during the reaction were removed by eluting the sample over Dowex 1-X8 as described by Linder et al. (1978). Horse heart myoglobin (type III) was obtained from Sigma and purified by the method of Tomoda et al. (1981). Protein solutions were dialyzed (Spectrapor No. 1 tubing) overnight at 4 °C against a 1500-fold excess of the appropriate cacodylate buffer and then centrifuged for 30 min at 5900*g* to remove small amounts of insoluble material. Methemoglobin was used within 27 h of oxidation. Concentrations of cytochrome b_5, methemoglobin, and metmyoglobin solutions were determined from published extinction coefficients (Ozols & Strittmatter, 1964; Mauk et al., 1982; Linder et al., 1978; Antonini & Brunori, 1971). Methemoglobin concentrations are given in terms of heme.

Glass-distilled water further purified by passage through a commercial deionizing train (Barnstead NANOpure) was used throughout. Cacodylic acid (Sigma, Nutritional Biochemicals) was recrystallized from absolute ethanol (Perrin et al., 1980). Buffers of known ionic strength were prepared by adding solid cacodylic acid to dilute solutions of sodium hydroxide (prepared from standardized stock solutions) to obtain the required pH as determined with a Radiometer Model PHM 84 meter and a combination electrode. Buffers were passed through a Millipore filter (0.45 μm) to remove particulate matter that might contribute to light scattering.

Difference spectra were obtained and spectrophotometric titrations were performed with a Cary 219 spectrophotometer and tandem cuvettes by the methods of Erman & Vitello (1980). Other instrumentation and data reduction procedures have been described (Mauk et al., 1982).

Results

The difference spectrum produced on mixing cytochrome b_5 and methemoglobin is shown in Figure 1. A difference

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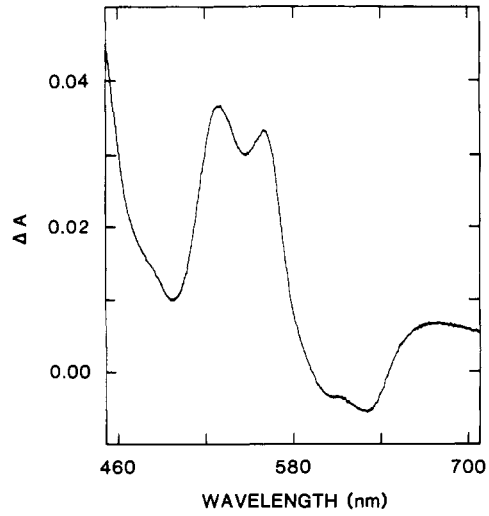

FIGURE 1: Difference spectrum resulting from interaction of cytochrome b_5 with methemoglobin (corrected for instrument base line). Tandem cuvettes being used, the reference cuvette contained 188.4 μM cytochrome b_5 in one compartment and 218.3 μM methemoglobin in the other, while the sample cuvette contained identical volumes (by mass) of the same solutions mixed with each other and placed in both sides of the cuvette [pH 6.2, μ = 5 mM (cacodylate buffer), 25 °C]. The cells were matched with path lengths of 0.874 cm.

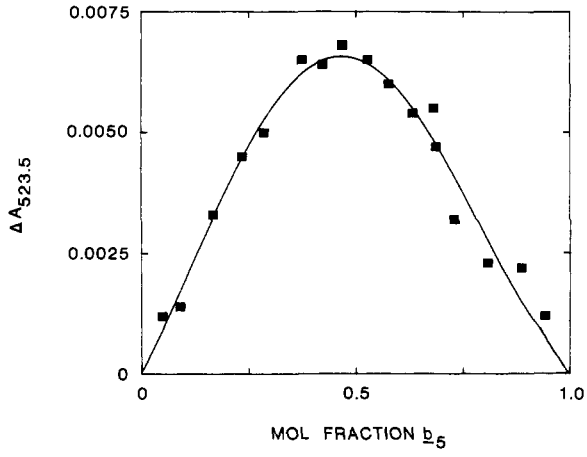

FIGURE 2: Job plot obtained by determining change in absorbance at 523.5 nm as a function of mole fraction of cytochrome b_5 and methemoglobin. The total concentration of the two proteins was held constant at 100.7 μM [pH 6.2, μ = 2 mM (cacodylate buffer), 25 °C]. The path length was 0.879 cm.

also occurs in the Soret region but cannot be characterized as well owing to loss of protein (primarily methemoglobin) on container walls at the low protein concentrations required for work in this region of the spectrum. As a small amount of oxyhemoglobin (6–14%) is produced by autoreduction of methemoglobin during the overnight dialysis and storage [similar to the reduction observed by Linder et al. (1978)], the extent of complex formation was monitored by determining the change in absorbance produced at 523.5 nm on mixing the two proteins. This wavelength is an isosbestic point for the spectra of oxyhemoglobin and methemoglobin. For compensation of this autoreduction, the amount of oxyhemoglobin present in each sample was subtracted from the total hemoglobin concentration to yield the true methemoglobin concentration. The change in absorbance illustrated in Figure 1 represents an increase of approximately 3.1% at 560 and 2.3% at 523.5 nm over the total absorbance prior to mixing. Significantly, repetition of this experiment under identical conditions with metmyoglobin replacing methemoglobin failed

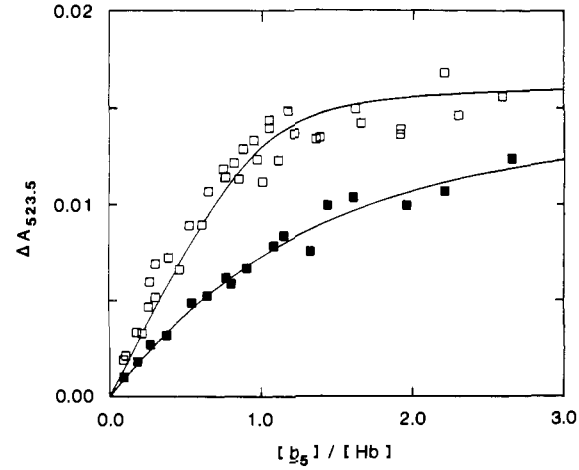

FIGURE 3: Spectrophotometric titrations of methemoglobin with increasing concentrations of cytochrome b_5. Methemoglobin concentration was 53 μM [pH 6.2 (cacodylate buffer), 25 °C]: (open squares) μ = 2 mM; (closed squares) μ = 7 mM. The path length was 0.879 cm.

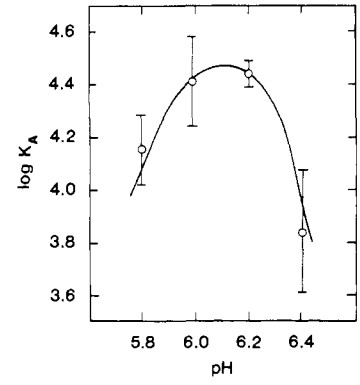

FIGURE 4: pH dependence of K_A for cytochrome b_5–methemoglobin complex formation [μ = 5 mM (cacodylate buffer), 25 °C].

to produce any evidence of a difference spectrum produced by mixing metmyoglobin and cytochrome b_5.

The stoichiometry of the complex formed between cytochrome b_5 and methemoglobin has been evaluated by the method of Job (1928) as shown in Figure 2. From this plot, it is apparent that a 1:1 (heme:heme) complex is produced under these conditions. This finding is corroborated by the titration curves shown in Figure 3 in which increasing concentrations of cytochrome b_5 were mixed with a constant concentration of methemoglobin. The association constants, K_A, determined from these plots are $(3 \pm 2) \times 10^5$ M^{-1} (μ = 2 mM) and $(3 \pm 1) \times 10^4$ M^{-1} (μ = 7 mM) (see paragraph at end of paper regarding supplementary material). From these measurements, the concentration of the cytochrome b_5–methemoglobin complex responsible for the spectrum shown in Figure 1 is 57.3 μM.

The pH dependence of cytochrome b_5–methemoglobin interaction is illustrated in Figure 4. As seen, the complex is most stable around pH 6.0–6.2 with K_A decreasing at more acidic or alkaline pH. Instability of the proteins prevented additional measurements at lower pH; at higher pH, this technique was insufficiently sensitive to measure binding.

Variation in binding was studied as a function of ionic strength as shown in Figure 5. As expected for the interaction between oppositely charged proteins, K_A was found to vary inversely with ionic strength. By use of the analytical approach of Wherland & Gray (1976) as employed by Geren & Millett (1981), these data have been fitted to the following relationship

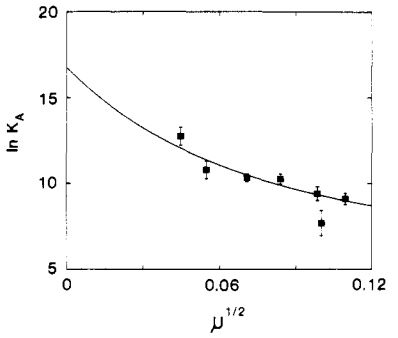

FIGURE 5: Variation of K_A for formation of complex between cytochrome b_5 and methemoglobin with ionic strength [pH 6.2 (cacodylate buffer), 25 °C]. The solid line is the weighted least-squares fit of the data to eq 1 performed as described in the text.

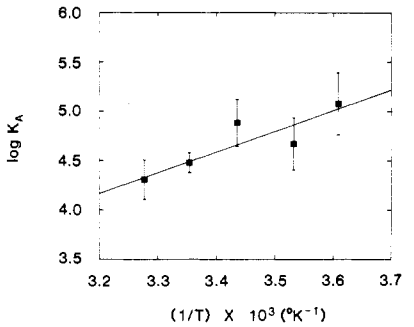

FIGURE 6: Variation in association constant for formation of cytochrome b_5–methemoglobin complex with temperature [pH 6.2 (cacodylate buffer), μ = 5 mM]. The solid line is a weighted linear least-squares fit of the data to the van't Hoff equation.

with the nonlinear least-squares program of Duggleby (1981) (Figure 5):

$$\ln K_A = \ln K_A^{\infty} - 3.576\left[\frac{e^{-\kappa R_1}}{1+\kappa R_2} + \frac{e^{-\kappa R_2}}{1+\kappa R_1}\right]\frac{Z_1Z_2}{R_1+R_2} \quad (1)$$

The charge product (Z_1Z_2) obtained from this analysis is −64 ± 14, in reasonable agreement with the value (−58.5) estimated from the sequences of the two proteins (Reid & Mauk, 1982; Mauk & Gray, 1979). The stability constant at infinite ionic strength is estimated to be 5×10^2 M^{-1}.

The thermodynamic parameters for cytochrome b_5–methemoglobin complex formation have been determined by measuring K_A over a range of temperatures and fitting the results to the van't Hoff equation with a weighted least-squares analysis (Figure 6). The results of this analysis yield $\Delta H°$ = −10 ± 3 kcal/mol and $\Delta S°$ = −12 ± 10 eu.

Discussion

Electron transfer reactions between metalloproteins are generally thought to proceed by means of a three-step process. The first step involves formation of a precursor complex wherein the active centers of the proteins are oriented in an optimal geometry with respect to each other to promote electron transfer. The second step is the transfer of the electron between metal centers to produce a successor complex. The final step is the dissociation of the successor complex to generate the products of the reaction. The present work and that of related reports on other pairs of metalloproteins [e.g., Poulos & Kraut (1980), Kraut (1981), Geren & Millett (1981), Erman & Vitello (1980), and Chiang (1981)] address the details of the first step, precursor complex formation. This process is of considerable interest because it is presumably responsible for the specificity of such reactions. In addition, the conditions under which precursor complex formation is optimal are useful information in the design of experiments to study the intramolecular electron transfer that occurs in the second step (Potasek & Hopfield, 1977a,b; Hopfield, 1977; Potasek, 1978). The present studies, of course, are limited by the inability to study the interaction of ferrocytochrome b_5 with methemoglobin at equilibrium. Nevertheless, it seems likely that stability constants determined here represent at least minimum values that would be observed if the ferrocytochrome b_5–methemoglobin interaction could be measured and that the effects of temperature, ionic strength, and pH are at least qualitatively similar.

A relatively clear initial picture of the cytochrome b_5–methemoglobin complex emerges from the results presented here. The most fundamental feature of this interaction is that each subunit of methemoglobin is able to bind one molecule of cytochrome b_5. The dependence of K_A on both ionic strength and pH indicates that this interaction involves electrostatic interactions between oppositely charged functional groups on the two proteins. This conclusion is consistent with the observed effect of ionic strength on the rate of methemoglobin reduction by methemoglobin reductase (Sannes & Hultquist, 1978), although ionic strength may have had additional effects in these earlier studies. From the structural information that is available for cytochrome b_5 and methemoglobin, it is most likely that carboxyl groups surrounding the partially exposed heme edge of cytochrome b_5 interact with basic residues near the heme pockets of the hemoglobin subunits.

With these considerations in mind, it should be possible to identify putative binding sites for cytochrome b_5 on hemoglobin α and β chains by examination of their three-dimensional structures for similar receptor sites. These cytochrome b_5 receptor sites should consist of lysyl and/or arginyl residues that are arranged in approximately the same orientation with respect to each other around the entrance to the heme pocket. In fact, five such lysyl residues can be identified in each subunit. For the α chain, these are residues 40, 56, 60, 61, and 90 while for the β chain they are residues 59, 61, 65, 66, and 95. Although other residues may be involved as well, these loci are probably the principal contact sites for cytochrome b_5 binding to hemoglobin. A related argument has been advanced by Gacon et al. (1980).

Although cytochrome b_5 reduces metmyoglobin (Hagler et al., 1979), no difference spectrum could be elicited on mixing these two proteins. This finding is consistent with the observation that none of the residues in myoglobin that correspond to the lysyl residues mentioned above are positively charged. We interpret this finding to mean that myoglobin and cytochrome b_5 do not form a stable complex. We cannot rule out the possibility that a complex forms without producing a difference spectrum, but this seems unlikely to us. As attempts to detect cytochrome b_5 in muscle tissue have been unsuccessful (Hagler et al., 1979), there is no apparent need for a cytochrome b_5 binding site on myoglobin. Thus, while complementary binding sites on two proteins for each other facilitate complex formation, they are not required for electron transfer to occur.

The thermodynamics of cytochrome b_5 interaction with methemoglobin are in marked contrast to those of cytochrome b_5 interaction with cytochrome c (Mauk et al., 1982). While the latter interaction was found to be dominated by entropic contributions, the former interaction falls more in line with

the model for protein–protein interaction proposed by Ross & Subramanian (1981) in which $\Delta H°$ and $\Delta S°$ are both negative. It seems likely that much of this difference arises from the ability of methemoglobin to undergo a significant change in conformation on complexation with cytochrome b_5. At least two mechanisms by which such a conformational change could promote electron transfer can be envisaged. First, a cytochrome b_5 induced change in methemoglobin conformation to a form more closely resembling deoxyhemoglobin would substantially reduce the reorganization energy barrier to electron transfer. Alternatively, induction of a hemichrome-like conformation could also be argued to promote electron transfer as reduction of a hemichrome to a hemochrome would entail oxidized and reduced active sites that are both low spin. Low-spin heme complexes are known to undergo more facile electron transfer than are high-spin complexes [e.g., Pasternack & Spiro (1978)]. At present, of course, direct evidence for a methemoglobin conformational change in this process is not available.

The role of solvent reorganization in complex formation is also difficult to assess. Perutz (1977) has indicated that water is bound internally to crystalline met- and deoxyhemoglobin primarily at the subunit interfaces with no change in the number of molecules bound occurring on interconversion of these two forms of the protein. However, these 90 internally bound water molecules represent only 10% of the total water unavailable as solvent to diffusible electrolytes (Adair & Adair, 1936). This remaining externally bound water is presumably the fraction that is more important in the interaction of hemoglobin with other proteins.

Three previous reports have addressed the problem of cytochrome b_5–methemoglobin interaction. Gacon et al. (1980) employed kinetic studies involving the enzymatic reduction of normal and mutant hemoglobins to implicate β-chain residues Lys-66 and Lys-95 in cytochrome b_5–methemoglobin contact. In addition, Gacon et al. (1980) and Righetti et al. (1978) have reported titration curves of mixtures of cytochrome b_5 and methemoglobin that they obtained by means of an isoelectric focusing technique. The results of these experiments were interpreted as indicating that complex formation is optimal in the pH range 8.0–8.3, at variance to our observations. One possible explanation for this discrepancy would be the interference of ampholyte–protein interaction in the previous studies. Finally, Juckett & Hultquist (1981) have observed a number of weak visible magnetic circular dichroism bands produced on mixing cytochrome b_5 with a variety of hemoglobin derivatives that they attribute to formation of a charge–transfer complex. These bands are presumably related to those present in the difference spectrum shown in Figure 1.

Acknowledgments

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Supplementary Material Available

A table of association constants (1 page). Ordering information is given on any current masthead page.

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Regeneration of Ribonuclease A from the Reduced Protein. Rate-Limiting Steps†

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ABSTRACT: In the regeneration of ribonuclease A (RNase A) from the reduced protein by a mixture of reduced and oxidized glutathiones, there is a distribution of intermediates in a preequilibrium state characterized by a set of equilibrium constants. In the kinetics experiments reported here, we measured the amounts of fully regenerated RNase A produced (in >50 min) from the reduced protein at various concentrations of reduced and oxidized glutathiones at pH 8.2 and 22 °C. We relate these amounts of fully regenerated RNase A to the distributions of the intermediates in preequilibrium in order to identify the rate-limitng steps and estimate their rate constants. Several different rate-limiting steps (or pathways) were found to produce fully regenerated RNase A. Also, by starting the regeneration from the isolated intermediates 4S (two or four of whose four disulfide bonds are nonnative ones) and 3S1G1H, we were able to suggest that the reaction 4S + GSH → 3S1G1H* is a more likely rate-limiting step than is 3S1G1H → 4S* + GSH. In summary, the regeneration of RNase A with glutathiones involves oxidation of reduced RNase A to a set of intermediates; interconversions among these intermediates rapidly lead to a preequilibrium state prior to the rate-limiting steps. At the same time, fully regenerated RNase A is produced from the intermediates (that exist in this preequilibrium state prior to the rate-limiting steps) through several *different* pathways (or rate-limiting steps). The preferred pathway that is followed varies according to the distribution of intermediates, i.e., according to the relative stabilities of the intermediates and the concentrations of reduced and oxidized glutathiones.

This series of papers presents a study of the regeneration of ribonuclease A (RNase A)[1] from the reduced protein by a mixture of oxidized and reduced glutathione (GSSG and GSH, respectively), which is being carried out within the context of the general problem of determining the pathways of protein folding from an analysis of the preequilibrium, the kinetics, and the energetics of the interconversion of the intermediates. In paper 3 (Konishi et al., 1981), we showed that there is a preequilibrium among the "Intermediates"[2] on the pathways toward fully regenerated RNase A, and we estimated the equilibrium constants (some of which were obtained by appropriate extrapolation) and distributions among the various intermediates in the preequilibrium.

Such a preequilibrium treatment is applicable to protein folding pathways in general. For example, in the folding of bovine pancreatic trypsin inhibitor, Creighton (1977) showed that the reaction involving disulfide interchange from [(30–51, 5–14) + (30–51, 5–38)] to (30–51, 5–55), in his notation, is the rate-limiting step. Thus, the reduced protein and the intermediates prior to the rate-limiting step should exist in a preequilibrium, and a similar preequilibrium treatment as that reported in paper 3 would be applicable to this system. A preequilibrium also exists in the folding of proteins with intact disulfide bonds. The kinetic parameters for folding of such systems have been obtained by jumping the solution conditions from denaturing ones (e.g., 8 M urea, 6 M guanidine hydrochloride, low pH, etc.) to folding conditions (Baldwin & Creighton, 1980). Within the dead time of the jump in solution conditions, the denatured proteins undergo very rapid conformational changes to "Intermediates" (Kato et al., 1981). Then, interconversions among the "Intermediates" lead to a preequilibrium distribution of these species; at the same time, the native conformation is attained through many possible pathways (rate-limiting steps) that can involve relatively fast and/or slow folding processes.

In this paper, we obtain data for the rate of formation of fully regenerated RNase A at various concentrations of GSSG and GSH, as was done to some extent by Hantgan et al. (1974) and Ahmed et al. (1975), and show that the preequilibrium treatment provides information about the rate-limiting steps, i.e., about the folding pathway(s). In paper 5, we analyze the

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[1] Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione; RNase A, bovine pancreatic ribonuclease A; BPTI, bovine pancreatic trypsin inhibitor; *l*S*m*G*n*H and *l*S*m*G*n*H*, intermediate prior to and after the rate-limiting steps, respectively, with *l* cystine residues, *m* mixed disulfide bonds between half-cystine and glutathione, and *n* free cysteine residues (if *l*, *m*, or *n* is equal to 0, *l*S, *m*G, or *n*H is omitted, e.g., 8H or 4S); Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CMC, carboxymethylcellulose.

[2] "Intermediates" and "Intermediates*" represent the intermediates prior to and after, respectively, the rate-limiting steps in the regeneration pathway of RNase A. The fully reduced protein is included in the "Intermediates". As shown in paper 3 (Konishi et al., 1981), there are 7192 intermediates on purely statistical grounds; undoubtedly, some of them involve steric hindrance and thus cannot exist.

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