Regeneration of Ribonuclease A from the Reduced Protein. Energetic Analysis†

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ABSTRACT: When ribonuclease A (RNase A) is regenerated from the reduced protein by a mixture of reduced and oxidized glutathiones, the intermediates and fully regenerated RNase A differ from each other in chemical composition. Hence, it is impossible to compare their total energies. Instead, the total chemical potential of each protein species is expressed in terms of an intrinsic chemical potential, which depends on the amino acid composition, and a conformational chemical potential, which depends on the conformation of the protein (whose entropy is influenced by the presence of disulfide bonds). The apparent standard state conformational chemical potentials of the "Intermediates" (relative to that of 4S taken as 0) were estimated by using data for the apparent equilibrium constants among these species and for the redox potentials of cysteine/cystine and reduced and oxidized glutathione. The relative standard state conformational chemical potential of native RNase A was estimated from the loss of chain entropy by formation of the native disulfide bonds. The results demonstrate that cleavage of the disulfide bonds stabilizes the disordered conformations, probably because of the increase of chain entropy. In the reduced protein, especially, the unfolded form is conformationally much more favorable than the native conformation. A general discussion of equilibrium and kinetic studies of protein folding is presented. Equilibrium data provide information about the *stabilities* of the conformational states of a protein and serve as the basis for an analysis of the distributions or relative stabilities of the intermediates that are present at preequilibrium prior to the rate-limiting step(s). On the other hand, kinetic data provide information about the *pathways* among these states, specifically about the rate-limiting step(s) along these pathways.

The energetics of conformational equilibria in proteins with *intact* disulfide bonds have been examined by calorimetric (Privalov, 1979) and spectroscopic (Hermans & Scheraga, 1961; Brandts & Hunt, 1967; Tanford, 1970; Contaxis & Bigelow, 1981) methods. On the other hand, in studying the regeneration of a protein from its *reduced* form, the energetic analysis is complicated both by the greater chemical complexity of the regeneration mixture and by the changes in chemical composition that accompany the formation of disulfide bonds in the course of regeneration.

In a study of the regeneration of bovine pancreatic trypsin inhibitor (BPTI)[1] from its reduced form, Creighton (1977b) assigned *apparent* free energies to the reduced, intermediate, and native species and computed the activation energies for their interconversions. Such a free energy scale, however, is not easily interpretable in terms of conformational changes that occur during folding because the various species to which the apparent free energies were assigned differ in chemical constitution, i.e., in their relative numbers of cystine and cysteine residues. To obtain information bearing directly on the conformation, it is necessary to eliminate the effect of differences in chemical constitution. Thus, Fukada & Takahashi (1980) estimated the conformational enthalpy change accompanying the reduction of the disulfide bonds in insulin by subtracting the enthalpy of bond breaking, i.e., the contribution from changes in chemical constitution. In this paper, we describe a general method to compute the standard state conformational chemical potentials of the various species encountered in the regeneration of a protein by oxidative formation of its disulfide bonds. It is developed, by using the regeneration of RNase A from its reduced form as an example, and then is also applied to the corresponding reactions for BPTI.

Analysis

Energetics for Regeneration of RNase A. In studying the regeneration of RNase A by glutathiones, we have established the existence of a preequilibrium state in which the fraction of each "Intermediate"[2] in the total population of "Intermediates" remains constant with regeneration time (Konishi et al., 1981). We also calculated the apparent equilibrium constants among the "Intermediates" in the preequilibrium state. It is now of interest to calculate the relative standard state *conformational* chemical potentials of all species in preequilibrium and compare them to that of native RNase A. Since these species differ in chemical composition, i.e., they differ in the numbers of RNase A(–SH), RNase A(–SSG) and RNase A(), we must take account

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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*, intermediates 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); RNase A(–SH), RNase A(–SSG), and RNase A() represent a cysteine residue, a half-cystine residue involved in a mixed disulfide bond with glutathione, and a cystine residue, respectively, in RNase A; DTT, dithiothreitol; DPN, diphosphopyridine nucleotide.

[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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of such differences in order to compute *conformational* chemical potentials.

For this purpose, we express the chemical potential μ of a protein in solution as a sum of two terms. One is an intrinsic chemical potential, μ_{int}, which depends on the amino acid composition (including bound glutathione) and may be estimated essentially as the sum of the chemical potentials of its constituent amino acid residues (Tanford, 1970). The other is the conformational chemical potential, μ_{conf}, which depends on the specific conformation of the protein molecule (which can be influenced by bound glutathione). Both μ_{int} and μ_{conf} include interactions with the solvent, which is water in the present case. It is the standard state value, μ^0_{conf}, for all species that is the quantity of interest here, because it is directly related to the interaction energies between the various atoms of the chain (in the given conformation in the given solvent); its estimation is described below.

We use "Intermediates" 4S and 3S1G1H as examples. The interconversion between 3S1G1H and 4S is represented by

$$3S1G1H \rightleftharpoons 4S + GSH \quad (1)$$

As discussed in paper 4 of this series (Konishi et al., 1982a), 3S1G1H and 4S are each ensembles of many species; thus, the equilibrium constant for eq 1 is an *apparent* one. At equilibrium, the apparent chemical potentials of reactants and products are equal, i.e.

$$\mu_{3S1G1H} = \mu_{4S} + \mu_{GSH} \quad (2)$$

Hence, since $\mu_i = \mu^0_i + RT \ln [x_i]$, i.e., approximating activities by mole fraction, x_i, and taking the standard state for all species as unit mole fraction

$$\mu^0_{3S1G1H} + RT \ln [3S1G1H] = \mu^0_{4S} + RT \ln [4S] + \mu^0_{GSH} + RT \ln [GSH] \quad (3)$$

The apparent standard state chemical potential, μ^0, may be expressed as the sum of the apparent standard state intrinsic chemical potential, μ^0_{int}, and the apparent standard state conformational chemical potential, μ^0_{conf}. Thus

$$(\mu^0_{conf})_{3S1G1H} - (\mu^0_{conf})_{4S} = (\mu^0_{int})_{4S} - (\mu^0_{int})_{3S1G1H} + \mu^0_{GSH} + RT \ln ([4S][GSH]/[3S1G1H]) \quad (4)$$

Since the only difference in the chemical composition of 4S and 3S1G1H is the presence in 4S of one cystine residue and the presence in 3S1G1H of one cysteine residue plus half of a cystine residue involved in a mixed disulfide bond with glutathione (all other amino acid residues being the same in both species), we may express the difference in intrinsic chemical potentials as

$$(\mu^0_{int})_{4S} - (\mu^0_{int})_{3S1G1H} = (\mu^0_{int})_{CySSCy} - [(\mu^0_{int})_{CySSG} + (\mu^0_{int})_{CySH}] \quad (5)$$

where the subscripts CySSCy, CySSG, and CySH refer to cystine, half-cystine involved in a mixed disulfide bond with glutathione, and cysteine, respectively. Then eq 4 becomes

$$(\mu^0_{conf})_{3S1G1H} - (\mu^0_{conf})_{4S} \simeq \mu^0_{CySSCy} - \mu^0_{CySSG} - \mu^0_{CySH} + \mu^0_{GSH} + RT \ln K_{3S1G1H:4S} \quad (6)$$

where $K_{3S1G1H:4S}$ is the apparent equilibrium constant (Konishi et al., 1982a) for reaction 1, which was obtained in Figure 1 of paper 3 (Konishi et al., 1981) as 5.4×10^{-5} (in mole fraction units), and the intrinsic chemical potentials of the residues (in this case, the redox potentials of the residues) are approximated by those of the free amino acids [because the redox potentials of GSH and free cysteine differ by only 0.016 V at neutral pH and room temperature (Jocelyn, 1967)]. The value of the first four terms on the right-hand side of eq 6 may be obtained from the following equilibrium between the free amino acids:

$$CySSCy + GSH \rightleftharpoons CySSG + CySH \quad (7)$$

At equilibrium

$$\mu^0_{CySSCy} + \mu^0_{GSH} = \mu^0_{CySSG} + \mu^0_{CySH} + RT \ln [[CySSG][CySH]/([CySSCy][GSH])] \quad (8)$$

From Jocelyn's (1967) measured value of $[CySSG][CySH]/([CySSCy][GSH]) = 3.0$ at neutral pH and 22 °C, which is independent of pH between pH 6 and 9 (Clark, 1960), we obtain

$$\mu^0_{CySSCy} - \mu^0_{CySSG} - \mu^0_{CySH} + \mu^0_{GSH} = RT \ln [[CySSG] \times [CySH]/([CySSCy][GSH])] = 642 \text{ cal/mol} \quad (9)$$

at 22 °C. Inserting this value and the value of $K_{3S1G1H:4S}$ into eq 6, we obtain

$$(\mu^0_{conf})_{3S1G1H} - (\mu^0_{conf})_{4S} = -5.1 \text{ kcal/mol} \quad (10)$$

at 22 °C and pH 8.2.

The values of the apparent $\Delta\mu^0_{conf}$ of all of the other intermediates were computed in a similar way from the apparent equilibrium constants in paper 3 (Konishi et al., 1981) and Jocelyn's (1967) data. Although some of the apparent equilibrium constants in paper 3 were obtained by extrapolation, they were used in this paper because their reliability was established in paper 4 (Konishi et al., 1982a). Taking $(\mu^0_{conf})_{4S}$ arbitrarily as 0, the values of μ^0_{conf} for the other "Intermediates" are represented in Figure 1.

Since the "Intermediates" are mostly denatured (Creighton, 1979; Konishi & Scheraga, 1980a,b; Galat et al., 1981), their relative values of μ^0_{conf} may be considered to result primarily

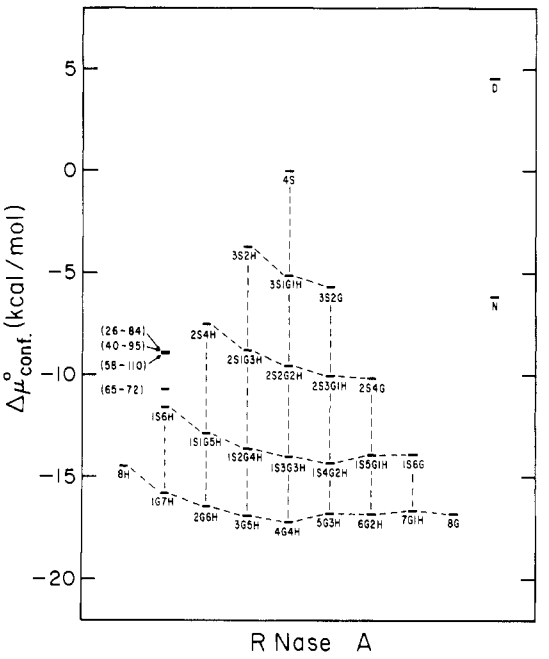

FIGURE 1: Apparent standard state conformational chemical potentials (relative to that of 0 for 4S) of "Intermediates" at 22 °C and pH 8.2. The horizontal and vertical dashed lines connect species that interconvert by reactions 1 and 2, respectively, in paper 3 (Konishi et al., 1981). Some of the apparent equilibrium constants in paper 3, used to estimate μ^0_{conf}, were obtained by extrapolation, but their reliability was established in paper 4 (Konishi et al., 1982a). The corresponding standard state conformational chemical potentials of native RNase A (N) and denatured RNase A with intact disulfide bonds (D), computed as described in the text, are plotted on the right side of the figure. $(\mu^0_{conf})_{(1S6H)_i}$ for species with *native* disulfide bonds are designated as (26–84), (40–95), (58–110), and (65–72) and are plotted above $(\mu^0_{conf})_{1S6H}$ (see text for details).

from four factors. One is due to conformational differences among the "Intermediates". The second is the reduction of the conformational entropy in the *unfolded* "Intermediates" by formation of disulfide bonds *nonrandomly* (Schellman, 1955; Flory, 1956; Poland & Scheraga, 1965; Anfinsen & Scheraga, 1975; Johnson et al., 1978). The third is another entropy contribution. Each "Intermediate" in Figure 1 is a mixture of many distinct molecules, which differ in the combinations of disulfide bonds and/or the residual numbers of cysteine or half-cystine residues involved in mixed disulfide bonds with glutathione (Konishi et al., 1981). Thus, the more species in an "Intermediate", the greater is its entropy, thereby the lower its value of μ^0_{conf}. The fourth arises from the incorporation of glutathiones. The bulkiness and one negative charge of glutathione would influence μ^0_{conf} of an "Intermediate" to which glutathione is bound.

We next consider the relative values of μ^0_{conf} for native RNase A (N) and for denatured RNase A with intact disulfide bonds (D) on the diagram of Figure 1. When we incubated native RNase A (7.3×10^{-5} M) with excess GSH (44 mM) at pH 8.2 and 22 °C for 2 weeks, which is a stronger reducing condition than those used in the regeneration experiments ([GSH] = 0.67–40 mM and [GSSG] = 0.4–24 mM; Konishi et al., 1982a), no reduced RNase A or intermediates were detected by carboxymethylcellulose column chromatography at pH 3.5 (Konishi et al., 1981); i.e., native RNase A is quite stable (kinetically) to reduction by GSH under these conditions (see footnote 3 of paper 4). Hence, the reactions from "Intermediates*" to "Intermediates" can reasonably be neglected under our experimental conditions in this series. Thus, since N is not involved in any measurably reversible equilibria at 22 °C, we cannot obtain μ^0_{conf} for N by the method used for the intermediates and must therefore obtain it by another method.

The entropy reduction due to overlapping disulfide-formed loops in a protein has been computed (Poland & Scheraga, 1965) and has been verified experimentally in a comparison of the thermal stabilities of native lysozyme and a cross-linked derivative of this protein (Johnson et al., 1978). Using this procedure, we estimate that μ^0_{conf} of the *denatured* form (D) is increased by about 19 kcal/mol over that of species 8H due to formation of the four correct disulfide bonds.[3] Since the conformation of reduced RNase A is mostly disordered (White et al., 1961; Tanford et al., 1966; Hantgan et al., 1974; Chavez & Scheraga, 1980b), μ^0_{conf} of the denatured conformation with four intact disulfide bonds would be 19 kcal/mol higher than $(\mu^0_{conf})_{8H}$. The free energy difference between N and D [which corresponds to $(\mu^0_{conf})_D - (\mu^0_{conf})_N$ because there is no chemical change upon denaturation, i.e., $(\mu^0_{int})_D = (\mu^0_{int})_N$] was measured as 10.7 kcal/mol at neutral pH and 22 °C by Privalov & Khechinashvili (1974) from an examination of the reversible denaturation of RNase A by variation of temperature and pH. Thus, we may write

$$(\mu^0_{conf})_N - (\mu^0_{conf})_D = -10.7 \text{ kcal/mol} \quad (11)$$

$$(\mu^0_{conf})_D - (\mu^0_{conf})_{8H} \simeq 19 \text{ kcal/mol} \quad (12)$$

By adding these two equations, we obtain

$$(\mu^0_{conf})_N - (\mu^0_{conf})_{8H} \simeq 8.3 \text{ kcal/mol} \quad (13)$$

Thus, μ^0_{conf} of the native structure is *not* the lowest among the species of Figure 1 but lies approximately between those of 3S2H and 2S4H, and that of the denatured form lies 10.7 kcal/mol higher (Figure 1).

In interpreting Figure 1, it must be emphasized that μ^0_{conf} of the "Intermediates" are the *apparent* relative standard state conformational chemical potentials, estimated from *apparent* equilibrium constants among the "Intermediates". Hence, μ^0_{conf} in Figure 1 does not correspond to the conformational chemical potentials of the 7191 individual intermediates (Konishi et al., 1981). However, μ^0_{conf} in Figure 1 is related to the standard state conformational chemical potentials of the individual species as follows. The "Intermediate" 1S6H, for example, is a mixture of 28 possible species, as mentioned in paper 4 of this series (Konishi et al., 1982a). Since they are in equilibrium with each other, we may write

$$\mu_{1S6H} = \mu_{(1S6H)_i} \quad (14)$$

where μ_{1S6H} and $\mu_{(1S6H)_i}$ are the apparent chemical potential of the "Intermediates" 1S6H and the chemical potential of one of the 28 possible species in 1S6H, respectively. Further, since 1S6H and $(1S6H)_i$ have the same chemical composition

$$(\mu_{int})_{1S6H} = (\mu_{int})_{(1S6H)_i} \quad (15)$$

Therefore

$$(\mu_{conf})_{1S6H} = (\mu_{conf})_{(1S6H)_i} \quad (16)$$

Hence

$$(\mu^0_{conf})_{(1S6H)_i} = (\mu^0_{conf})_{1S6H} - RT \ln [[(1S6H)_i]/[1S6H]] \quad (17)$$

Thus, it is clear that μ^0_{conf} of an *individual* intermediate is higher than that shown in Figure 1 because $[(1S6H)_i] < [1S6H]$.

The difference between $(\mu^0_{conf})_{(1S6H)_i}$ and $(\mu^0_{conf})_{8H}$ may be estimated by assuming that it arises from the entropy loss due to formation of a disulfide bond.[3] The estimated values of $[(\mu^0_{conf})_{(1S6H)_i} - (\mu^0_{conf})_{8H}]$ for the 28 possible species $(1S6H)_i$ lie in the range of 5930–3750 cal/mol, and the values of $(\mu^0_{conf})_{(1S6H)_i}$ for the four species containing native disulfide bonds are plotted in Figure 1 as examples. The relative population of each intermediate was calculated from

$$(\mu^0_{conf})_{(1S6H)_i} - (\mu^0_{conf})_{8H} = (\mu^0_{conf})_{(1S6H)_j} - (\mu^0_{conf})_{8H} - RT \ln [[(1S6H)_i]/[(1S6H)_j]] \quad (18)$$

Since the differences in (μ^0_{conf})'s on both sides of eq 18 were estimated by calculating the entropy loss due to formation of a disulfide bond, the values of $[(1S6H)_i]$ could be computed by arbitrarily taking $[(1S6H)_j]$ as 1.0. Then a value of [1S6H] was computed from

$$[1S6H] = \sum_{i=1}^{28} [(1S6H)_i] \quad (19)$$

Insertion of this value into the following modification of eq 17:

$$(\mu^0_{conf})_{1S6H} - (\mu^0_{conf})_{8H} = (\mu^0_{conf})_{(1S6H)_i} - (\mu^0_{conf})_{8H} + RT \ln [[(1S6H)_i]/[1S6H]] \quad (20)$$

leads to a value of 2770 cal/mol for $[(\mu^0_{conf})_{1S6H} - (\mu^0_{conf})_{8H}]$. This value, obtained by estimating the entropy loss, agrees very well with the value of 2880 cal/mol of Figure 1, obtained

[3] Poland & Scheraga (1965) treated the entropy loss when polypeptide chains are multiply cross-linked in overlapping (dependent) loops and estimated that the introduction of three correct disulfide bonds in specific locations in native RNase A (the small loop, Cys-65–Cys-73, was excluded) reduced the entropy of the chain by about 49–52 eu. An estimate by S. Ihara (personal communication) for *four* correct disulfide bonds [using the method of Wang & Uhlenbeck (1945) with the parameters of Poland & Scheraga (1965)] led to a value of 64 eu. At 22 °C, the latter corresponds to an increase in free energy of 19 kcal/mol.

directly from the apparent equilibrium constants.

Another point is that Figure 1 pertains only to the *conformational* chemical potentials of the various intermediates. However, the relative populations of each species in preequilibrium are functions of not only μ^0_{conf} but also μ^0_{int}, $\mu^0_{glutathione}$, and the concentrations of the glutathiones, as shown in eq 4. Therefore, Figure 1 by itself does not provide any information about the relative populations of "Intermediates" and fully regenerated RNase A, shown (later) in Figure 4.

Finally, Figure 1 provides a justification for an assumption made in paper 3 (Konishi et al., 1981) to compute some of the equilibrium constants that could not be measured directly. There it was assumed that the equilibrium constant for the reaction in which one cystine residue is formed from two cysteine residues depends on the number of cystine residues in any of the intermediates and is independent of the number of bound glutathiones. This assumption was based on the expectation that μ^0_{conf} would be influenced primarily by the number of disulfide bonds (i.e., by the dominance of the chain entropy) and not by the number of bound glutathiones. Figure 1 demonstrates that this assumption is approximately valid since the *almost*-horizontal dashed lines connect species with the same number of disulfide bonds but with different numbers of bound glutathiones.

Energetics for Regeneration of BPTI. Creighton (1977b) measured the apparent equilibrium constants involved in the regeneration reactions from reduced BPTI with DTT. We apply the foregoing treatment and analyze his equilibrium constants to estimate μ^0_{conf} of the reduced, intermediate, and native forms of BPTI, as was done for RNase A. In this analysis, we use *Creighton*'s notation for the protein and for oxidized and reduced DTT.

For this purpose, we need the analogue of eq 7 and 8 for the reaction between DTT and cysteine/cystine. The equilibrium constant for this reaction has been measured indirectly (Cleland, 1964) from data for reactions between DPN and DTT, and cysteine, respectively. Since the data for the latter reaction were not as accurate as those for the former (W. W. Cleland, private communication) and the reported reduction potentials of cysteine at pH 7 have a large uncertainty, ranging from −0.14 to −0.40 V (Clark, 1960), some of which may arise from use of two different concentration units (moles per liter and mole fraction) (Rost & Rapoport, 1964), we have instead taken the equilibrium constant for the reaction between DTT and cysteine/cystine from data of Creighton (1977b) as follows. The equilibrium constant for the reaction

$$N^{SH}_{SH} + DTT^{S}_{S} \rightleftarrows N^{S}_{S} + DTT^{SH}_{SH} \qquad (21)$$

was measured by Creighton (1977b) as 0.15 at 25 °C, where N^{SH}_{SH} contains two native disulfide bonds and the nativelike conformation (Creighton, 1978; Creighton et al., 1978; Kosen et al., 1980), N^{S}_{S} is fully regenerated BPTI, and DTT^{S}_{S} and DTT^{SH}_{SH} are the oxidized and reduced forms, respectively, of DTT. At equilibrium

$$(\mu^0_{int})_{N^{SH}_{SH}} + (\mu^0_{conf})_{N^{SH}_{SH}} + RT \ln [N^{SH}_{SH}] + \mu^0_{DTT^{S}_{S}} + RT \ln [DTT^{S}_{S}] = (\mu^0_{int})_{N^{S}_{S}} + (\mu^0_{conf})_{N^{S}_{S}} + RT \ln [N^{S}_{S}] + \mu^0_{DTT^{SH}_{SH}} + RT \ln [DTT^{SH}_{SH}] \qquad (22)$$

Since

$$(\mu^0_{int})_{N^{SH}_{SH}} - (\mu^0_{int})_{N^{S}_{S}} \simeq 2\mu^0_{CySH} - \mu^0_{CySSCy} \qquad (23)$$

as in eq 6, and

$$(\mu^0_{conf})_{N^{SH}_{SH}} \simeq (\mu^0_{conf})_{N^{S}_{S}} \qquad (24)$$

because N^{SH}_{SH} has a conformation very close to the native one, i.e., N^{S}_{S}, eq 22 [with the aid of the equilibrium constant of eq 21 (=0.15)] becomes

$$2\mu^0_{CySH} - \mu^0_{CySSCy} + \mu^0_{DTT^{S}_{S}} - \mu^0_{DTT^{SH}_{SH}} \simeq RT \ln [[N^{S}_{S}][DTT^{SH}_{SH}]/([N^{SH}_{SH}][DTT^{S}_{S}])] = -1.12 \text{ kcal/mol} \qquad (25)$$

Equation 25 also applies to the following equilibrium between cysteine/cystine and DTT:

$$2CySH + DTT^{S}_{S} \rightleftharpoons CySSCy + DTT^{SH}_{SH} \qquad (26)$$

for which we may approximate K as 0.15.

With the value of $K = 0.15$ for reaction 26, we have estimated $\Delta\mu^0_{conf}$ of reduced, intermediate, and native BPTI, as we have done for RNase A. For example

$$R + DTT^{S}_{S} \rightleftarrows I + DTT^{SH}_{SH} \qquad (27)$$

where R and I are the reduced and intermediate forms, respectively, where I contains one disulfide bond. At equilibrium

$$(\mu^0_{conf})_R - (\mu^0_{conf})_I = \mu^0_{CySSCy} - 2\mu^0_{CySH} + \mu^0_{DTT^{SH}_{SH}} - \mu^0_{DTT^{S}_{S}} + RT \ln [[I][DTT^{SH}_{SH}]/([R][DTT^{S}_{S}])] \qquad (28)$$

Creighton (1977b) reported the equilibrium constants among the various species of BPTI, regenerated with DTT; with the aid of the equilibrium constant (=8.7×10^{-4}) for reaction 27 and the value given in eq 25

$$(\mu^0_{conf})_R - (\mu^0_{conf})_I = 1.12 \times 10^3 - 4.18 \times 10^3 = -3060 \text{ cal/mol} \qquad (29)$$

In a similar way, $\Delta\mu^0_{conf}$'s of other species of BPTI were estimated, and the results are shown in Figure 2 by arbitrarily taking $(\mu^0_{conf})_N$ as 0.

We may rationalize our choice of $K = 0.15$ for reaction 26 as follows. A similar analysis is applicable to the oxidation of reduced RNase A by DTT^{S}_{S}, for which Creighton (1977c)

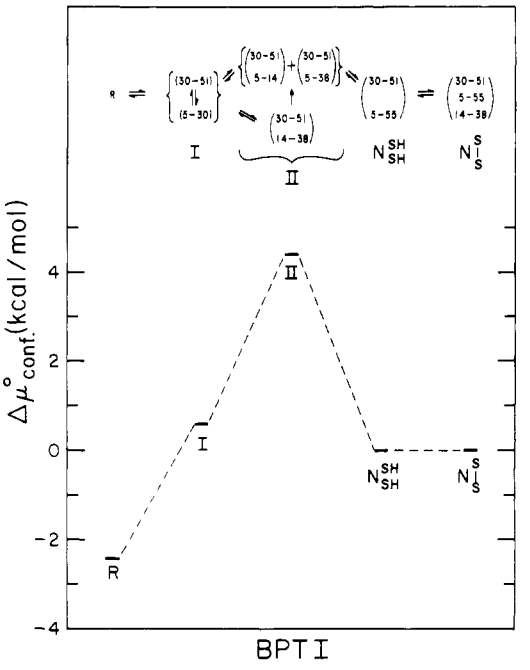

FIGURE 2: Relative standard state conformational chemical potentials of reduced, intermediate, and native forms of BPTI, computed from experimental data reported by Creighton (1977b). The standard state conformational chemical potential of native BPTI is arbitrarily taken as 0. The reaction scheme and notation are those of Creighton (1977b). I and II designate mixtures of the dominant intermediates, [(30–51) and (5–30)] and [$\binom{30-51}{5-14}$, $\binom{30-51}{5-38}$, and $\binom{30-51}{14-38}$], respectively. Therefore, their values of μ^0_{conf} are *apparent* ones. (5–30), (5–38), and (5–14) are incorrect pairings.

reported equilibrium constants of 1.3×10^{-3} and 4.0×10^{-4} for reactions 30 and 31, respectively.

$$8H + DTT^{S}_{S} \rightleftharpoons 1S6H + DTT^{SH}_{SH} \quad (30)$$

$$1S6H + DTT^{S}_{S} \rightleftharpoons 2S4H + DTT^{SH}_{SH} \quad (31)$$

With these equilibrium constants and with $K = 0.15$ for reaction 26, we compute

$$(\mu^0_{conf})_{1S6H} - (\mu^0_{conf})_{8H} = 2770 \text{ cal/mol} \quad (32)$$

and

$$(\mu^0_{conf})_{2S4H} - (\mu^0_{conf})_{1S6H} = 3480 \text{ cal/mol} \quad (33)$$

which agree with the values of 2880 and 4060 cal/mol, respectively, in Figure 1. On the other hand, if we use Cleland's (1964) equilibrium constant (0.0036, dimensionless, computed from his value for concentration in mole per liter), instead of 0.15, for reaction 26, then the values in eq 32 and 33 become 610 and 1308 cal/mol, respectively. Since the conformations of 8H, 1S6H, and 2S4H are mostly denatured (Creighton, 1979; Konishi & Scheraga, 1980a,b; Galat et al., 1981), the differences in conformational chemical potential in eq 32 and 33 should be large and positive because of the reduction in entropy due to formation of a disulfide bond (3–5 kcal/mol for loops in proteins). Thus, the values computed with $K = 0.15$ are more reasonable than those computed with 0.0036. Even with the larger value of 0.15, DTT is still a strong reducing agent in the cysteine/cystine system; e.g., the value of 0.15 corresponds to the following equilibrium concentrations: [CySH] = 1 mM, [CySSCy] = 2.7×10^{-3} mM, $[DTT^{S}_{S}] = [DTT^{SH}_{SH}] = 1$ mM, and $[H_2O] = 55.5$ M. These values, when converted to mole fractions, give $K = 0.15$; i.e., CySSCy is reduced.

Discussion

Equilibrium and Kinetic Studies. In studying the pathways of protein folding, two different approaches, viz., an equilibrium one and a kinetic one, have been developed. In this section, we discuss the information obtained by these two approaches. Figure 3 demonstrates the difference between the two techniques. No chemical reaction such as oxidation/reduction is involved in Figure 3; thus, $\Delta\mu^0_{conf} = \Delta\mu^0$ because μ^0_{int} is the same for all species. Figure 3 shows, as an example, the values of μ^0_{conf} for simple folding/unfolding pathways plotted against the degree of order/disorder, where the degree of order of the protein conformation increases as it passes from the completely unfolded to the completely folded form. Since the equilibrium technique can detect intermediates only in the transition region, the same values are taken for μ^0_{conf} of the native conformation, N, and the denatured conformations, D, in Figure 3. We assume that there are two pathways, A and B, between N and D containing only one relatively stable intermediate. Pathway A has a stable intermediate, IA, but the activation energies between N and IA and between IA and D are assumed to be large. Pathway B has a relatively unstable intermediate, IB, but the activation energies between N and IB and between IB and D are low compared to those of pathway A. Under these assumptions, the equilibrium technique detects the energetically stable species, N, IA, and D. Thus, the pathway in the equilibrium study would be taken to be

$$N \rightleftharpoons IA \rightleftharpoons D \quad (34)$$

i.e., pathway A. Since the kinetic technique follows the process with the lowest activation energy, the resulting pathway is

$$N \rightleftarrows IB \rightleftarrows D \quad (35)$$

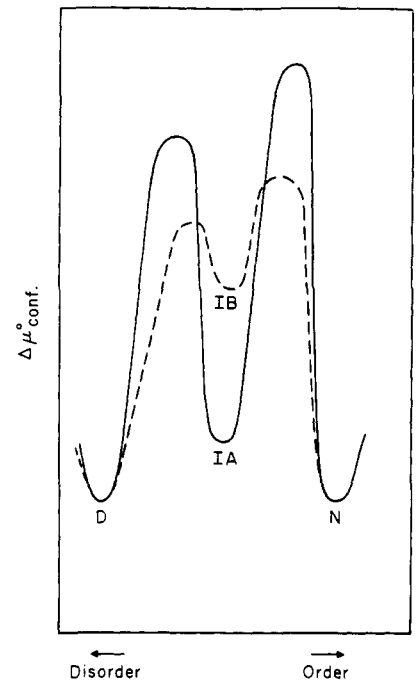

FIGURE 3: Schematic representation of two hypothetical conformational folding/unfolding pathways for a protein without any chemical reaction (such as oxidation/reduction), A (solid line) and B (dashed line), in terms of μ^0_{conf} vs. order/disorder of the protein conformation. N and D represent the native and denatured conformation, respectively. IA and IB correspond to relatively stable intermediates in pathways A and B, respectively. The peak heights between N or D and the intermediates correspond to the activation energies between the states.

demonstrating that the dominant pathway between N and D is pathway B. Figure 3 clearly shows that a kinetic study provides the correct information about the pathway. The equilibrium study provides information about the stability of each conformation or state but does not provide the pathway between the states.

We next expand the discussion to the regeneration from reduced RNase A, consider the possibility that an equilibrium among all species including N exists, and ask whether we can obtain information about the folding pathway(s) from the hypothetical equilibrium studies. Even though such an equilibrium state could not be attained experimentally, because the back reaction from fully regenerated RNase A is too slow to detect (when glutathione is used as the reducing agent), we may use the relative values of μ^0_{conf} (Figure 1), μ^0 for CySSCy, CySH, GSH, and GSSG, and the mole fractions of GSH and GSSG to compute the populations of the "Intermediates" and native RNase A in equilibrium (as in eq 6). The result is shown in Figure 4. When RNase A is equilibrated with various concentrations of GSH (4.2×10^{-5}–0.11 M) and a fixed concentration of GSSG (4.2×10^{-5} M), native RNase A and 8H are the most oxidized and reduced species, respectively, and 1S6H and 1G7H are detectable intermediates (more than 1% of the population). But the pathway should not be described as

$$N \rightleftarrows 1S6H \rightleftarrows 1G7H \rightleftarrows 8H \quad (36)$$

because states 1G7H and 1S6H are populated (stable) only in the transition region, under *equilibrium* conditions. The folding/unfolding of RNase A, however, proceeds *kinetically* under any solution conditions, i.e., folding, transition, or unfolding conditions. For example, at high concentrations of GSH (and [GSSG] = 4.2×10^{-5} M), RNase A is regenerated from 8H through the pathway "1S6H" and, at relatively low concentrations of GSH (and [GSSG] = 4.2×10^{-5} M),

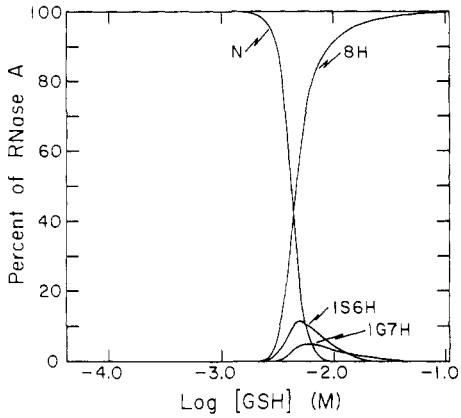

FIGURE 4: Calculated populations (%) of "Intermediates" and native RNase A in the *hypothetical* equilibrium with [GSH] in the range of 4.2×10^{-5}–0.11 M and [GSSG] = 4.2×10^{-5} M at pH 8.2 and 22 °C. The populations of the other intermediates were negligible (<1%).

through the pathways "2S1G3H", "3S1G1H", "2S2G2H", and "3S2H", as shown in paper 4 (Konishi et al., 1982a). Thus, although 1G7H and 1S6H are intermediates on the regeneration pathways, reaction 36 does not represent the pathway for the regeneration of RNase A.

In some cases, however, the regeneration pathway observed in kinetic studies could coincide with one based on stable intermediates. This may be the situation for the pathway, deduced by Burgess & Scheraga (1975) and modified by Matheson & Scheraga (1978) and Chavez & Scheraga (1980a), for the equilibrium thermal unfolding of RNase A with intact disulfide bonds. The first two sets of workers deduced the pathway by considering the stable intermediates (in equilibrium) at various temperatures. As cited by Scheraga (1980), a variety of subsequent physicochemical measurements were in agreement with this pathway, and further evidence for it was provided recently by X-ray crystal structure studies of RNase A at various temperatures (G. Petsko, private communication). Chavez & Scheraga (1980a) interpreted their kinetic data[4] by suggesting that the Burgess/Matheson/Scheraga pathway for *thermal* unfolding might be applicable to the pathway for regeneration by glutathiones. Thus, in some cases, the results of an equilibrium study and a kinetic study might be consistent, as they appear to be for BPTI (Creighton, 1978) and RNase A (Chavez & Scheraga, 1980a). But, it should be emphasized that, in general, equilibrium techniques provide information about the stabilities of the states but not about the pathways.

In papers 3 and 4 (Konishi et al., 1981, 1982a), we have carried out preequilibrium and kinetic studies of the regeneration of RNase A. We have shown that the "Intermediates" should be analyzed by a preequilibrium rather than a kinetic treatment, because the "Intermediates" rapidly attain a *preequilibrium* state; further, conversion from the "Intermediates" to fully regenerated RNase A, i.e., the rate-limiting steps, should be analyzed by a kinetic treatment since the rate-limiting steps are not involved in equilibria because of the negligible amount of the back (unfolding) reactions under unfolding (and, hence, certainly under folding) conditions. Thus, both preequilibrium and kinetic studies provide useful information about protein regeneration, but it must be recognized that each type of study yields a different (but related) kind of information.

Regeneration Pathways of BPTI. In this section, we discuss the regeneration pathways of BPTI from the viewpoint of the standard state conformational chemical potential of the protein. In the ensemble of reduced species, a certain fraction has a nativelike conformation and, hence, the same value of μ^0_{conf} as the native protein, viz., 2.4 kcal/mol higher than that for reduced BPTI at 25 °C and pH 8.7 (Figure 2). (Since the protons on sulfhydryl groups are very small, there is essentially no steric hindrance preventing reduced BPTI from taking on a nativelike conformation, with the same value of μ^0_{conf}.) Therefore, according to the Boltzmann distribution, about 1.7% of reduced BPTI would be expected to have a nativelike conformation at equilibrium. Thus, one of the possible regeneration pathways, which we have designated elsewhere as a "growth-type" pathway (Konishi et al., 1982b), would be the following:

$$R_D \rightleftharpoons R_N \rightarrow I_N \rightarrow II_N \rightarrow N \qquad (37)$$

where R_D and R_N are reduced forms of BPTI in the disordered and nativelike conformations, respectively, and I_N, II_N, and N are species (all of which have nativelike conformations) with one, two, and three disulfide bonds, respectively. The detailed kinetic study by Creighton (1977a), however, did not detect pathway 37. If R_N were formed, there would not seem to be any barrier for it to convert rapidly to I_N (especially by formation of a disulfide bond between Cys-14 and Cys-38 in the nativelike conformation). Since I_N was not detected and II_N was formed in a pathway other than eq 37 (because pathway 37 was not detected), we have to assume that little R_N was produced, probably because of the high activation barrier between R_D and R_N. Therefore, eq 37 is not a folding pathway for BPTI; i.e., this protein does not fold along a "growth-type" pathway, as originally concluded by Creighton (1978).

Another interesting point in Figure 2 is that, if the kinetically-trapped intermediates with native disulfide bonds [(30–51) and (30–51, 14–38)] had the same conformation as the native protein, they would have the same values of μ^0_{conf}. Figure 2, however, shows that μ^0_{conf} for the native protein is lower than that for both species I and II. This demonstrates that [(30–51) and (30–51, 14–38)] do not have the native conformation, presumably because there are high activation barriers or topological differences that prevent them from reaching the native conformation (Scheraga, 1981). Thus, the regeneration of BPTI prior to the rate-limiting step may be characterized by high activation energies between the unfolded and nativelike conformations. It seems that such large potential barriers are reduced by formation of an incorrect disulfide bond(s), 5–14 or 5–38, and reshuffling from the incorrect bond to the native one, 5–55. In another paper (Konishi et al., 1982b), we have designated this type of regeneration as a "rearrangement-type" pathway and have discussed it in detail.

Regeneration Pathways of RNase A. The rate-limiting step for RNase A, which corresponds to that for BPTI [viz., (30–51, 5–14) or (30–51, 5–38) → (30–51, 5–55)], would be the reshuffling of disulfide bonds from 3S2H to 3S2H*, because both [(30–51, 5–14) or (30–51, 5–38)] in BPTI and 3S2H in RNase A have two cysteine residues, no bound DTT or glutathiones, and a higher value of μ^0_{conf} than that of the native conformation; and the reshuffling of a disulfide bond(s) is the rate-limiting step in both pathways. Paper 4 of this series (Konishi et al., 1982a) indicated that this process, from 3S2H

[4] Chavez & Scheraga (1980a) used a lower pH (7.0) than that (pH 8.2) used in the experiments in paper 4 (Konishi et al., 1982a). Since the pH dependence of the apparent equilibrium constants is not known and no consideration was given to the question of the attainment of a preequilibrium state in the experiments of Chavez & Scheraga (1980a), we cannot compare our kinetic data to theirs.

to 3S2H*, can be one of the rate-limiting steps in the regeneration of RNase A. Also, the accumulated intermediate III_n of RNase A, in Creighton's notation, which may correspond to 3S2H* in our notation (Konishi et al., 1982b) even though it did not accumulate under our experimental conditions (Konishi et al., 1982a), might correspond to N^{SH}_{SH} of BPTI because both have three or two correct disulfide bonds, respectively (lacking one disulfide bond), and nativelike conformations (Creighton, 1980; Galat et al., 1981). But the pathway with the rate-limiting step from 3S2H to 3S2H*, by itself, cannot account for the kinetic data of paper 4, and it seems that RNase A is also regenerated through other pathways. Therefore, a single pathway (involving two different intermediates), as observed for BPTI, is not the only one for protein folding, and in general, a protein may be regenerated through *many* pathways.

Conformations of Proteins. The value of μ^0_{conf} pertains to the conformation of the *whole* protein molecule. Thus, it is possible that denatured proteins may take on nativelike conformations in a limited *local* portion of the molecule. For example, an immunological assay of reduced RNase A showed that about 6% of a nativelike conformation existed at four antigenic sites (Chavez & Scheraga, 1980b). This means that some (native) antigenic sites, but not all four sites simultaneously, exist (to the extent of about 6%) in *each* molecule.

Finally, we consider the strong thermodynamic driving force that directs a protein to adopt its native conformation. First of all, the disulfide bonds contribute to the stability. Taking RNase A as an example, the most stable form of its cysteine/cystine residues at low concentrations of GSH and GSSG is cystine, i.e., 4S and native RNase A, as mentioned in paper 3 (Konishi et al., 1981). In addition, intramolecular interactions contribute to the stability of the native conformation. Figure 1 shows that $(\mu^0_{conf})_N - (\mu^0_{conf})_{4S} = -6.1$ kcal/mol; hence $\mu^0_N - \mu^0_{4S} = -6.1$ kcal/mol because $(\mu^0_{int})_N = (\mu^0_{int})_{4S}$. Thus, the native conformation is the most stable form (i.e., the native protein is more stable than 4S) in an equilibrium system at low concentrations of GSH and GSSG. In other words, the conformation of native RNase A *in water* (i.e., with no GSH or GSSG present) is indeed the one of minimum free energy; i.e., the native protein is *not* trapped in a metastable state. Since GSH and GSSG stabilize the RNase A(–SH) and RNase A(–SSG) forms, respectively (Konishi et al., 1981), 8H becomes the most stable form in the presence of excess GSH and 8G becomes the most stable form in the presence of excess GSSG (see Figure 4 of this paper and Figure 7 of paper 3).

Conclusions

The chemical potential of a protein can be expressed in terms of an intrinsic and a conformational chemical potential. In this way, it is possible to provide an energetic analysis of proteins that differ in chemical composition (i.e., in cysteine/cystine content). Such an analysis was carried out for two examples, RNase A and BPTI. In both proteins, the native structure is not at a minimum in the conformational chemical potential. However, the population of each species depends not only on its conformational chemical potential but also on its environment. The strong thermodynamic driving force that directs a protein to adopt its native conformation arises from the facts that (1) the intramolecular disulfide is the most stable form of cysteine/cystine under weak reducing and oxidizing conditions and (2) of all the disulfide forms (4S + N), the native conformation has the lowest value of μ^0_{conf}, i.e., is the most stable. We also showed that the regeneration of a proteir can be studied by a preequilibrium treatment, which provides information about the stabilities or distributions of "Intermediates", and by a kinetic method, which can provide information about the rate-limiting steps.

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Observation of Intermediates in the Folding of Ribonuclease A at Low Temperature Using Proton Nuclear Magnetic Resonance†

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ABSTRACT: The refolding of ribonuclease A (RNase A) has been investigated in aqueous methanol cryosolvents in the 0 to −20 °C range. When a thermally unfolded sample was brought under renaturing conditions (e.g., −16 °C, 35% methanol, pH* 2.8), the refolding, as monitored by the absorbance change at 286 nm (which reflects the degree of solvent exposure of Tyr), was triphasic and took approximately 1 h for completion. The 360-MHz proton nuclear magnetic resonance (NMR) spectrum of the native enzyme in either 35% or 50% aqueous methanol is very similar to that in aqueous solution. When the refolding of RNase A was monitored in the subzero temperature range with the signals of the His C2 protons, new resonances rapidly appeared, in addition to those from native protein. The new resonances are attributed to a partially folded intermediate state that has a relatively compact structure. Time-dependent changes were observed in the areas of the resonances from both native and partialy folded species. The rates of peak area reduction for the intermediate state were the same as those for the increase in area of the native resonances, and similar to those for the second phase observed in the absorbance experiments. The results are consistent with the slow-refolding form of RNase A consisting of a least two distinct populations. A model for the folding of RNase A is proposed.

Recent evidence suggests that the folding of small proteins such as RNase A[1] is intermediate controlled (Cook et al., 1979; Schmid, 1981; Schmid & Baldwin, 1979; Blum et al., 1978); that is, it is determined by the structure and stability of partially folded intermediate states (Ptitsyn & Rashin, 1975). The present study was undertaken to shed light on the nature of such intermediate species.

The refolding of RNase A has been extensively studied, especially by Baldwin and co-workers [e.g., Baldwin (1975, 1980) and Baldwin & Creighton (1980)], under a variety of conditions in aqueous solutions. Evidence for at least two different intermediates has been reported. On the basis of competition experiments between amide proton exchange and folding, Schmid & Baldwin (1979) demonstrated the presence of an early intermediate, lacking significant compact, globular structure. An extensive series of experiments by Cook et al. (1979) and Schmid (1981; Schmid & Blaschek, 1981) has shown that a nativelike intermediate, I_N, is formed during folding under "strongly" native conditions. Pro-93 has been proposed to be in the nonnative trans configuration in I_N. This intermediate can bind specific inhibitors, is catalytically active, and is presumed to have a structure virtually identical with that of the native protein except in the immediate vicinity of the loop around Pro-93.

The proton NMR spectrum of a protein contains a large amount of potential structural information. If partially folded intermediates are present during refolding, new transient resonances may be observed whose chemical shifts are different from those of the unfolded and native states. In the present study we have focused on the four His C2 protons since their resonances are well resolved and separated from those of other protons. There is one previous report in which NMR has been used to monitor the kinetics of the refolding of RNase A (Blum et al., 1978). In this instance RNase A was heated to 46 °C, pH 2.1, and rapidly quenched to 10 °C, and spectra were collected at 1-min intervals. A new resonance, in addition to those from unfolded and native states, was detected and attributed to the C2 proton of His-12 in an environment similar to that in the S peptide. It was concluded that formation of the N-terminal helix (residues 3–13) occurred at an early stage in refolding, prior to the formation of significant structure about the remaining three His residues.

We have shown that *methanol-based cryosolvents at subzero temperatures have no adverse effects on the catalytic and structural properties of RNase A* (Fink & Grey, 1978; R. G. Biringer and A. L. Fink, unpublished results; A. L. Fink, D. Kar, and R. Kotin, unpublished results). Experiments at subzero temperatures have been shown to be valuable in the detection of normally transient intermediates in enzyme catalysis (Fink, 1977; Douzou, 1977; Fink & Cartwright, 1981). Preliminary experiments have indicated that such an approach might also be very useful in protein folding studies (Fink & Grey, 1978).

We have recently used high-resolution proton NMR to monitor the unfolding of RNase A in the presence of methanol (R. G. Biringer and A. L. Fink, unpublished results). The investigation revealed that partially folded intermediates, in

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[1] Abbreviations: RNase, ribonuclease; NMR, nuclear magnetic resonance; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; N, native; U, unfolded; δ, chemical shift; S/N, signal to noise ratio; Gdn·HCl, guanidine hydrochloride; 2′,3′-cCMP, cytidine cyclic 2′,3′-phosphate; 2′-CMP, cytidine 2′-phosphate.

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