Structure and Stability of Insulin Dissolved in 1-Octanol

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Abstract: Addition of stoichiometric amounts of sodium dodecyl sulfate (SDS), an anionic detergent, to aqueous solutions of insulin produces a SDS–insulin complex which has limited solubility in water. However, the complex displays markedly enhanced solubility in nonaqueous solvents, such as 1-octanol. Concentrations of up to 1 mg/mL of insulin in 1-octanol can be obtained. Using circular dichroism spectroscopy, it was found that insulin maintains its native-like secondary and tertiary structure in 1-octanol. Insulin can be extracted back into an aqueous phase from 1-octanol, again with retention of its native conformation, provided there is sufficient chloride present to displace the bound detergent molecules. The stability of insulin with respect to thermal denaturation is significantly increased, as the T_m is shifted by 50 °C relative to unfolding in water.

I. Introduction

Factors governing the structure and stability of proteins continue to be an important focus of biophysical chemistry. However, our knowledge of protein structure is almost entirely based upon the behavior of proteins in aqueous media. Recently, it has been shown that enzymes suspended in organic solvents do display altered activity and specificity.[1-6] In addition to suspensions, microemulsions and reverse micelles have been employed as alternative approaches for placing enzymes in low-water environments.[7-10] Unfortunately, methods for truly dissolving chemically unmodified proteins in nonpolar solvents have been lacking. To a large degree, insolubility in organic solvents is partly due to the significant charge that most proteins possess at a given pH. If it were possible to mask that charge and increase the overall hydrophobicity, altered solubility characteristics might be achieved.

It has been known for some time that proteins and polypeptides can interact specifically with small amounts of anionic detergents,[11-18] such as sodium dodecyl sulfate (SDS), provided that the polypeptide possesses some degree of positive charge.[19] At low concentrations of SDS, it has been shown that the aqueous solubility of a protein is markedly diminished.[11-18] It is believed that this occurs as the result of ion pairing between the negatively charged detergent and the positively charged side chains of arginine and lysine, a phenomenon referred to as hydrophobic ion pairing (HIP). Upon further addition of SDS, the protein can be resolubilized into water through micelle formation. However, until this work, the solubility, structure, and stability of these HIP complexes in nonaqueous solvents have not been examined. It should be emphasized that the amounts of SDS employed in these experiments are well below the critical micelle concentration (cmc); thus, the behavior is distinct from that observed for micellar systems.

II. Materials and Methods

Apparent partition coefficients were measured by dissolving the peptide in 1.25 mL of an aqueous solution. Before SDS addition, the pH was measured on a Beckman pH meter. Typically, the pH of the insulin solution was approximately 2.5. Upon addition of a given volume of a concentrated SDS solution, the mixture turned cloudy and a precipitate formed immediately. An equal volume of 1-octanol was added, and the mixtures were agitated and then left undisturbed for several hours. Prior to analysis, the tubes were spun for 10 min at 4000*g*. Each layer was removed, and the absorbance was measured on a Beckman DU-64 UV-visible spectrophotometer using 1-cm quartz cells. All apparent partition coefficients were corrected for changes in pH with differing SDS concentrations, and all SDS:insulin ratios are given on a molar basis. Concentrations were determined assuming that the molar extinction coefficient was independent of the solvent.

Zinc-containing porcine insulin was obtained from either Sigma (St. Louis) or Eli Lilly (Indianapolis). A 1 mg/mL solution of zinc insulin in water absorbs 1.05 (path length 1 cm).[20] A molecular weight of 5808 was used in all calculations. Both SDS and 1-octanol were obtained from Sigma.

All circular dichroism (CD) spectra were measured using an Aviv 62DS spectrophotometer equipped with a thermoelectric temperature control unit. All temperatures were measured to ±0.2 °C. Samples were placed in 1-mm strain-free quartz cuvettes and equilibrated prior to any sample measurement. A bandwidth of 1 nm and an averaging time of 3 s were used for all the spectra reported here.

Melting curves for insulin–SDS complexes were determined by monitoring the molar ellipticity at 222 nm. Samples were heated at either 0.5 or 1.0 °C increments, and the signals at each temperature were averaged over 3 s. The T_m value was obtained by taking the first derivative of the melting curve.

III. Results and Discussion

Insulin is a small protein comprising two polypeptide chains totaling 51 amino acid residues, whose structure, stability, and activity have been widely investigated. Monomeric insulin contains six basic groups (two histidines, one arginine, one lysine, and two N-terminal amino groups) and six acidic groups (four glutamate residues and two C-terminal carboxylates). Near its isoelectric point (p*I*) of 5.5, insulin is quite insoluble. However, by lowering the pH to 2.5, all of the acidic groups become protonated, the only charge remaining on the protein is from the basic functionalities, and significant solubility in water is obtained.

Upon addition of stoichiometric amounts of SDS (1 SDS per basic group), the aqueous solubility of insulin is lowered dramatically, yielding a precipitate (Figure 1). Conversely, while the solubility of insulin in water decreases, its solubility in organic phases increases, producing a marked overall increase in the

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(1) Klibanov, A. M. *CHEMTECH* **1986**, *16*, 471-489.
(2) Klibanov, A. M. *Acc. Chem. Res.* **1990**, *23*, 114-120.
(3) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 3194-3201.
(4) Zaks, A.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 8017-8021.
(5) Russell, A. J.; Klibanov, A. M. *J. Biol. Chem.* **1988**, *263*, 11624-11626.
(6) Gupta, M. N. *Eur. J. Biochem.* **1992**, *203*, 25-32.
(7) Mattiasson, B.; Aldercreutz, P. *Trends Biotechnol.* **1991**, *9*, 394-398.
(8) Luisi, P. L.; Laane, C. *Trends Biotechnol.* **1986**, *4*, 153-161.
(9) Martinek, K.; Klibanov, A. M.; Goldmacher, V. S.; Berezin, I. V. *Eur. J. Biochem.* **1986**, *155*, 453-468.
(10) Waks, M. *Proteins: Struct., Funct., Genet.* **1986**, *1*, 4-15.
(11) Putnam, F. W.; Neurath, H. *J. Am. Chem. Soc.* **1944**, *66*, 692-698.
(12) Markus, G.; Karush, F. *J. Am. Chem. Soc.* **1957**, *79*, 3264-3269.
(13) Oakes, J. *Eur. J. Biochem.* **1973**, *36*, 553-558.
(14) Oakes, J.; Cafe, M. C. *Eur. J. Biochem.* **1973**, *36*, 559-563.
(15) Satake, I.; Tang, J. T. *Biochem. Biophys. Res. Commun.* **1973**, *54*, 930-936.
(16) Braude, I. A.; De Clerq, E. *J. Biol. Chem.* **1979**, *254*, 7758-7764.
(17) Hegg, P.-O. *Biochim. Biophys. Acta* **1979**, *579*, 73-87.
(18) Moskaitis, J. E.; Campagnoni, A. T. *Neurochem. Res.* **1986**, *11*, 299-315.
(19) Igou, D. K.; Lo, J.-T.; Clark, D. S. *Biochem. Biophys. Res. Commun.* **1974**, *60*, 140-145.
(20) Brems, D. N.; Brown, P. L.; Heckenlaible, L. A.; Frank, B. *Biochemistry* **1990**, *29*, 9289-9293.

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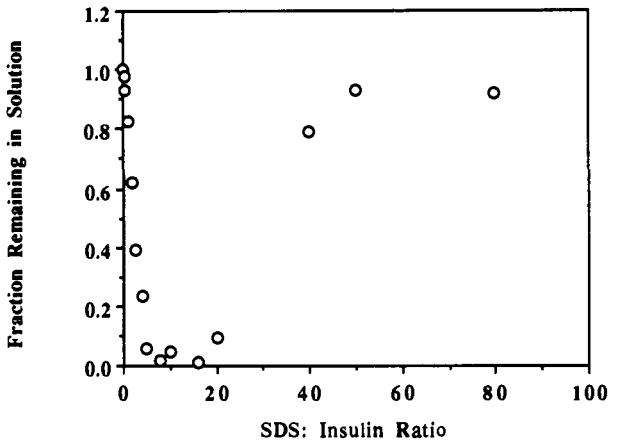

Figure 1. Dependence of insulin solubility upon concentration of SDS. The pH of the insulin solution was 2.5, and the SDS:insulin ratios are given on a molar basis.

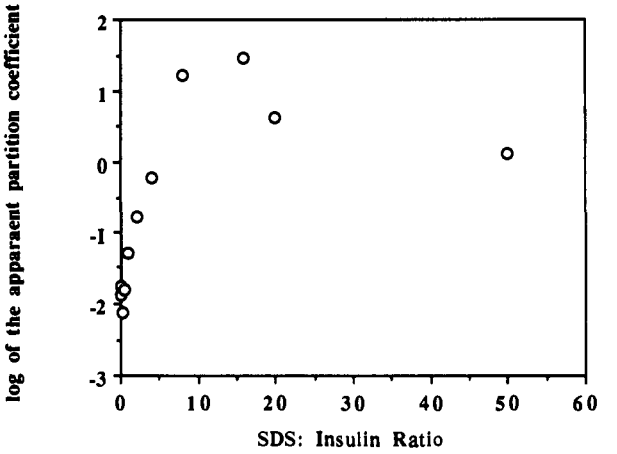

Figure 2. Logarithm of the apparent partition coefficient between 0.005 M HCl and 1-octanol. Prior to analysis, the tubes were spun for 10 min at 4000*g*. Each layer was removed, and the absorbance was measured on a Beckman DU-64 UV-visible spectrophotometer using 1-cm quartz cells. All apparent partition coefficients were corrected for changes in pH with differing SDS concentrations.

apparent partition coefficient. For example, partitioning into 1-octanol increases by almost 4 orders of magnitude upon addition of SDS (Figure 2). This represents the first successful approach toward the dissolution of chemically unmodified proteins in nonpolar solvents without the use of carrier systems (reverse micelles, microemulsions).

The behavior described here is fundamentally different from that for systems containing high concentrations (above the cmc) of SDS, such as are used in electrophoresis. For example, in order to form a stoichiometric complex at an insulin concentration of 1 mg/mL (6:1 SDS–insulin), the SDS concentration is less than 1 mM, significantly below the cmc, which at low ionic strengths is near 5 mM.[21,22] Further addition of SDS does lead to increased aqueous solubility (Figure 1), as well as a decreased apparent partition coefficient (Figure 2), presumably due to micelle formation.

In neat 1-octanol, the solubility of SDS–insulin complexes approaches 3 mg/mL. Without SDS, the solubility of insulin in 1-octanol is below the detection limits of the methods employed here (<0.03 mg/mL). In addition, SDS–insulin complexes have been found to be soluble in other organic solvents, such as *N*-methylpyrrolidinone (NMP), 2-propanol, and trimethyl phosphate (TMP). However, they were not found to be soluble in alkanes or chlorocarbon solvents (Table I). Apparently, some degree of solvent polarity is needed to solubilize HIP protein complexes.

Table I. Partitioning and Solubility Behavior of Insulin in Nonaqueous Solvents

SDS ratio[a]	organic solvent	log *P*[b]	app sol.[c]
3:1	1-octanol	≥0.8	≥1.0
6:1	1-octanol	≥1.2	≥3.0
6:1	CCl_4	nd[e]	insoluble[d]
6:1	mineral oil	nd	insoluble
6:1	CH_2Cl_2	nd	insoluble
6:1	hexane	nd	insoluble
6:1	1-chlorooctane	nd	insoluble
6:1	ether	nd	insoluble
6:1	THF[f]	miscible	na[g]
6:1	acetone	miscible	na
6:1	DMF	miscible	≥0.90
6:1	NMP	miscible	≥0.90
6:1	ethyl acetate	miscible	insoluble
6:1	acetonitrile	miscible	insoluble
6:1	dioxane	miscible	insoluble
6:1	PEG 400	miscible	≥0.14
6:1	trimethyl phosphate	miscible	≥0.90
6:1	*tert*-butyl alcohol	miscible	≥0.05
6:1	ethanol	miscible	≥0.90
6:1	2-propanol	miscible	≥0.90
6:1	propylene glycol	miscible	≥0.90
6:1	trifluoroethanol	miscible	≥0.90

[a] Ratio is of SDS to polypeptide. [b] Logarithm of the apparent partition coefficient. [c] Apparent solubility. Units are mg/mL. Molarity can be obtained by dividing by 5808. [d] Insoluble means that the solubility is lesss than 0.005 mg/mL. [e] Not detectable. No amount of insulin could be detected in either phase by absorbance spectroscopy. [f] Abbreviations: THF = tetrahydrofuran; NMP = *N*-methylpyrrolidinone; TMP = trimethyl phosphate; DMF = *N*,*N*-dimethylformamide; PEG = polyethylene glycol. [g] Not assessed. The absorbance of the solvent was too large to accurately determine the presence of insulin.

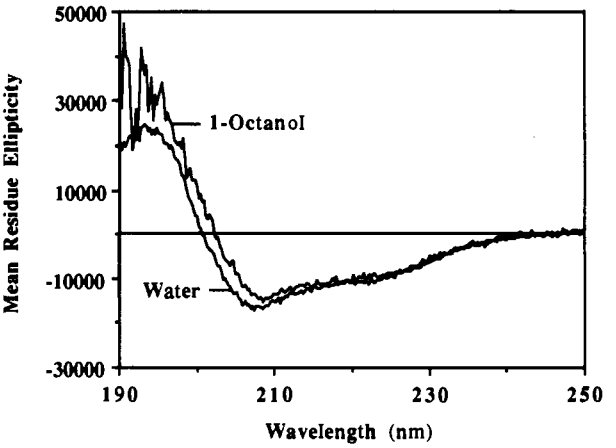

Figure 3. Far-UV CD spectra of 6:1 SDS–insulin complex in 1-octanol (50 μg/mL) and of insulin in water (pH 2.5, 50 μg/mL). All CD measurements were made on an Aviv 62DS circular dichroism spectrophotometer equipped with a thermoelectric temperature control device. The path length was 1 mm, and the sample temperature was 25 °C.

Although the discovery of the ability to dissolve insulin in nonpolar solvents using SDS is an important finding, it is essential that the properties of the SDS–insulin complex be determined. One consideration is whether the structure of the protein is retained when it is dissolved in a nonpolar medium. Using CD spectroscopy, one can show that the native secondary structure of the SDS–insulin complex in neat 1-octanol is similar to that of insulin in aqueous solution (Figure 3). Analysis of the CD data[23,24] indicates an α-helix content of 57%. This is in good agreement with results from insulin in aqueous solution (35–57%)[25–27] and

(21) Makino, S.; Niki, R. *Biochim. Biophys. Acta* **1977**, *495*, 99–109.
(22) Emerson, M. F.; Holzer, A. *J. Phys. Chem.* **1967**, *71*, 1898–1907.
(23) Johnson, W. C., Jr. *Proteins: Struct., Funct., Genet.* **1990**, *7*, 205–214.
(24) van Stokkum, I. H. M.; Spoelder, H. J. W.; Bloemendal, M.; van Grondelle, R.; Groen, F. C. A. *Anal. Biochem.* **1990**, *191*, 110–118.
(25) Ettinger, M. J.; Timasheff, S. N. *Biochemistry* **1971**, *10*, 824–831.
(26) Pocker, Y.; Biswas, S. B. *Biochemistry* **1980**, *19*, 5043–5049.

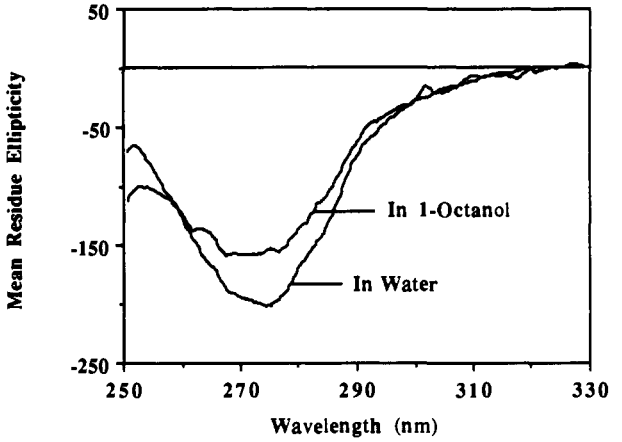

Figure 4. Near-UV CD spectra of zinc-containing porcine insulin in water (0.667 mg/mL, pH 2.5) and in 1-octanol (6:1 SDS-insulin, 0.667 mg/mL). The path length was 1 cm, and the sample temperature was 27 °C.

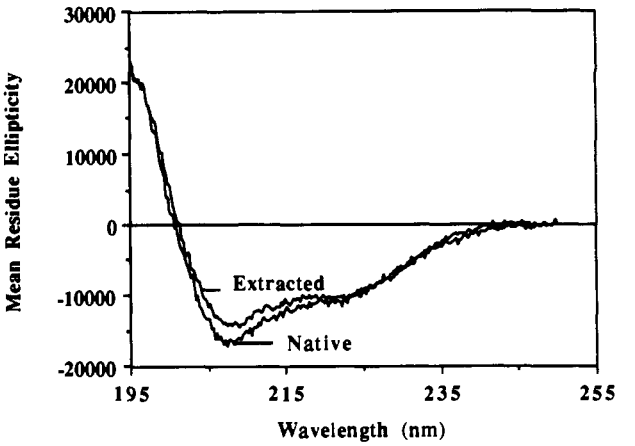

Figure 5. Far-UV CD spectrum of insulin extracted from 1-octanol into 0.100 M HCl. The concentration of insulin was 53 μg/mL, the path length was 1 mm, and the sample temperature was 5 °C. For comparison, the far-UV CD spectrum of porcine insulin (50 μg/mL, pH 2.5) taken under identical conditions is shown.

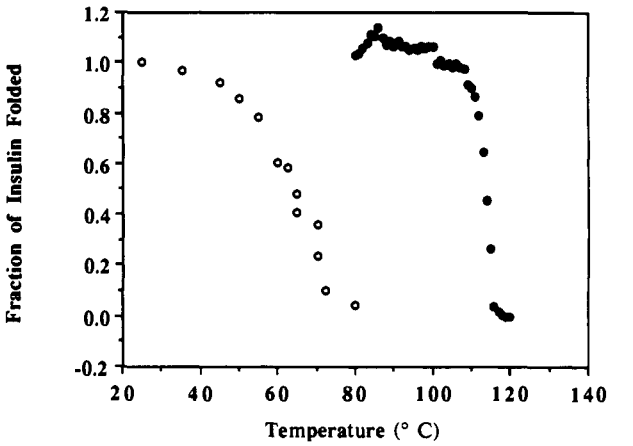

Figure 6. Thermal denaturation curve for insulin dissolved in 1-octanol (●) and in pH 3 water (○). The data for denaturation in water was taken from ref 22.

from crystalline insulin (53%), as determined by X-ray crystallography.[28] The CD spectra in 1-octanol are slightly more intense than those reported for insulin in water (Figure 3),[25-27] while the relative intensities of the CD bands are similar to those observed for insulin at high concentrations in water.[25] Together, these data indicate that the secondary structure composition is similar, with possibly only slightly greater α-helix content for the SDS–insulin complex.

Similarity in secondary structure composition does not assure that the SDS–insulin complex adopts a native-like structure in 1-octanol. Molten globule states also exhibit secondary structures similar to the native conformation, but lack any organized tertiary structure.[29] Molten globules do not display any appreciable CD intensity in the near-UV. Native insulin in water displays a strong negative near-UV CD band centered near 278 nm. The near-UV CD intensity of insulin is dependent on the aggregation state, which is a function of the pH, the zinc content, and the protein concentration. Examination of the near-UV CD reveals that the SDS–insulin complex in 1-octanol displays a spectrum similar to that of insulin in water (Figure 4). In both cases, porcine zinc insulin was used at identical concentrations (0.667 mg/mL). The CD of insulin in 1-octanol is less intense than in water, suggesting that the insulin molecules are in a lower state of aggregation, as the intensity is comparable to that of modified insulins[30,31] and of zinc-free insulin at low concentrations.[26,32,33] These data indicate not only that HIP insulin retains its native structure but also that it is probably monomeric in solution.

Again, the effect of hydrophobic ion pairing with anionic detergents is markedly different from that observed at high SDS concentrations. For example, large amounts of SDS are known to disrupt protein tertiary structure while stabilizing certain secondary structural features such as α-helices.[34,35] Yet, low concentrations of SDS appear to only slightly alter the overall conformation of insulin. It is clear that complexation with stoichiometric amounts of SDS can radically enhance the solubility of a protein in organic solvents without disrupting its structure.

The exact nature of the interaction between insulin and SDS is unknown. Presumably, it is governed by both electrostatic and hydrophobic interactions. It is known that SDS binds at very specific high-affinity sites on bovine serum albumin,[20,36-38] and the possibility exists that such sites could be found on insulin. If so, it should be possible to bind SDS reversibly, having SDS being replaced by another counterion, such as chloride. If this hypothesis is correct, agitating a solution of insulin in 1-octanol with an aqueous solution containing sufficient amounts of chloride should lead to extraction of insulin into the aqueous phase. In order to assess the reversibility of the partitioning into 1-octanol, a 1-octanol solution of 6:1 SDS–insulin was shaken with an equal volume of water containing 0.10 M HCl for 1 min. Replacement of the SDS counterions with chloride occurs, and insulin is extracted back into the aqueous phase. The far-UV CD spectrum of insulin extracted from 1-octanol into water is very similar to that of native insulin (Figure 5).

The stability of insulin to thermal denaturation is difficult to assess, as chemical degradation rates are rapid at elevated tem-

(27) Melberg, S. G.; Johnson, W. C., Jr. *Proteins: Struct., Funct., Genet.* **1990**, *8*, 280–286.
(28) Baker, E. N.; Blundell, T. L.; Cutfield, J. F.; Cutfield, S. M.; Dodson, E. J.; Dodson, G. G.; Crowfoot Hodgkin, D. M.; Hubbard, R. E.; Isaacs, N. W.; Reynolds, C. D.; Sakabe, K.; Sakabe, N.; Vijayan, N. M. *Philos. Trans. R. Soc. London* **1988**, *B319*, 369–456.
(29) Goto, Y.; Fink, A. L. *Biochemistry* **1989**, *28*, 945–952.
(30) Brange, J.; Ribel, U.; Hansen, J. F.; Dodson, G.; Hansen, M. T.; Havelund, S.; Melberg, S. G.; Norris, F.; Norris, K.; Snel, L.; Sørensen, A. R.; Voigt, H. O. *Science* **1988**, *333*, 679–682.
(31) Bryant, C.; Strohl, M.; Green, L. K.; Long, H. B.; Alter, L. A.; Pekar, A. H.; Chance, R. E.; Brems, D. N. *Biochemistry* **1992**, *31*, 5692–5698.
(32) Goldman, J.; Carpenter, F. H. *Biochemistry* **1974**, *13*, 4566–4574.
(33) Morris, J. W. S.; Mercola, D. A.; Arquilla, E. R. *Biochim. Biophys. Acta* **1968**, *160*, 145–150.
(34) Jordi, W.; Li-Xin, Z.; Pilon, M.; Demel, R. A.; de Kruijff, B. *J. Biol. Chem.* **1989**, *264*, 2292–2301.
(35) Snel, M. M. E.; Kaptein, R.; de Kruijff, B. *Biochemistry* **1991**, *30*, 3387–3395.
(36) Halfman, C. J.; Nishida, T. *Biochim. Biophys. Acta* **1971**, *243*, 294–303.
(37) Tanenaka, O.; Aizawa, S.; Tamaura, Y.; Hirano, J.; Inada, Y. *Biochim. Biophys. Acta* **1972**, *263*, 696–703.
(38) Nozaki, Y.; Reynods, J. A.; Tanford, C. *J. Biol. Chem.* **1974**, *249*, 4452–4459.
(39) Volkin, D.; Staubli, A.; Langer, R.; Klibanov, A. M. *Biotechnol. Bioeng.* **1991**, *37*, 843–853.

peratures. The only report in the literature regarding the unfolding of insulin is by Ettinger and Timasheff,[24] although it is likely that the process was not fully reversible due to chemical decomposition. The melting transition in water is broad, having a T_m near 63 °C. In 1-octanol, the SDS-insulin complex appears to still possess its native structure, even after prolonged heating at 70 °C (>60 min). Thermal denaturation of insulin in 1-octanol displays a high degree of cooperativity. The T_m of the 6:1 SDS-insulin complex in 1-octanol has been measured, by following molar ellipticity at 222 nm, and it occurs at ~115 °C (Figure 6), almost 50 °C above that observed in water! In addition, the T_m appears to be a function of SDS concentration, with optimal stabilization occurring in the same range as maximal apparent partitioning into 1-octanol. Therefore, it appears that proteins dissolved in nonpolar solvents may demonstrate exceptional thermal stability.

Similar increases in T_m have been observed for ribonuclease suspended in nonane versus samples in aqueous solution.[38] The T_m of ribonuclease is increased by approximately 35 °C. The lack of water in the lyophilized protein may account for its increased stability. Presumably, HIP complexes contain a similar amount of water to that of a lyophilized protein.

Unfortunately, the thermal denaturation of insulin is not reversible, preventing a thermodynamic determination of the increase in stability.[40] When heated past the T_m, the insulin rapidly precipitates from solution. One possible explanation would be that the limited amount of water available cannot adequately solubilize the expanded volume of the unfolded state, which displays essentially no solubility in 1-octanol.

(40) Pace, C. N. *Trends Biotechnol.* **1990**, *8*, 93-98.

IV. Summary

Hydrophobic ion pairing represents a powerful technique for obtaining solutions of peptides and proteins in nonaqueous solvents. Complex formation involves stoichiometric addition of an anionic detergent to an aqueous solution of a protein or peptide, specific binding of the detergent to the charged residues, and precipitation from solution. This material can be redissolved in a number of organic solvents, including such nonpolar media as 1-octanol. In 1-octanol, the native structure of the protein appears to be maintained, and the material can be partitioned back into an aqueous phase, again with no loss of structure. The thermal denaturation of insulin in 1-octanol is significantly retarded, and due to the lack of water, increased chemical stability would also be predicted.[6] The applicability of hydrophobic ion pairing to other systems, especially enzymes, is being investigated. These complexes may offer distinct advantages in the delivery of therapeutically important peptides.

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S-Aryl-L-cysteine *S*,*S*-Dioxides: Design, Synthesis, and Evaluation of a New Class of Inhibitors of Kynureninase

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Abstract: The design, preparation, and evaluation of *S*-aryl-L-cysteine *S*,*S*-dioxides, a new class of potent competitive inhibitors of kynureninase from *Pseudomonas fluorescens*, are described. The most potent of these compounds, *S*-(2-aminophenyl)-L-cysteine *S*,*S*-dioxide, has a K_i value of 70 nM. These analogues form prominent visible absorption peaks at 500 nm, assigned to quinonoid intermediates, when bound to kynureninase. Titration of kynureninase with *S*-(2-aminophenyl)-L-cysteine *S*,*S*-dioxide demonstrates that 1 mol of the inhibitor is bound to the pyridoxal 5'-phosphate in each subunit. Comparative molecular field analysis of the effects of structural variation on inhibitory potency allows us to predict that the (*S*)-*gem*-diolate anion of L-kynurenine, a proposed reaction intermediate, binds with a K_D of 19 nM. These results provide strong additional support for the intermediacy of a *gem*-diol or *gem*-diolate anion in the reaction mechanism of kynureninase.

Introduction

Kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) is a pyridoxal 5'-phosphate (PLP) dependent enzyme which catalyzes the conversion of L-kynurenine to L-alanine and anthranilic acid.[1] Kynureninase plays an important role in the catabolism of L-tryptophan in *Pseudomonas* and some other bacteria. In animals and plants, a similar enzyme, 3-hydroxykynureninase, is involved in the metabolism of L-tryptophan to L-alanine and 3-hydroxyanthranilic acid, which is further metabolized to quinolinic acid, and finally to NAD.[1] Recent research has suggested that quinolinic acid is neurotoxic and may be involved in the etiology of neurodegenerative diseases such as Huntington's chorea, epilepsy, and AIDS-related dementia.[2-4] Thus, potent and selective inhibitors of this enzyme could be of value in the treatment of these diseases. There are only a few reported inhibitors for this enzyme, including β-chloroalanine and *S*-(*o*-nitrophenyl)-L-cysteine,[5] which are "suicide substrates". However, these compounds are relatively toxic, and they inactivate a large number of PLP-dependent enzymes. In our previous publication,[6] we have reported that (4*S*)- and (4*R*)-dihydro-L-kynurenine are potent competitive inhibitors of kynureninase, with K_i values of 0.3 μM and 1.4 μM, respectively.

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(1) Soda, K.; Tanizawa, K. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1979**, *49*, 1.
(2) (a) Schwarz, R.; Okuno, E.; White, R. J.; Bird, E. D.; Whetsell, W. O. *Proc. Natl. Acad. Sci., U.S.A.* **1988**, *85*, 4079. (b) Beal, M. F.; Kowall, N. W.; Ellison, D. W.; Mazurck, M. F.; Swartz, K. J.; Martin, J. B. *Nature* **1986**, *321*, 168.
(3) Mazzari, S.; Aldinio, C.; Beccaro, M.; Toffano, G. J.; Schwarz, R. *Brain Res.* **1986**, *380*, 309.
(4) Heyes, M. P.; Brew, B. J.; Martin, A.; Price, R. W.; Salazar, A. M.; Sidtis, J. J.; Yergey, J. A.; Mouradian, M. M.; Sadler, A. E.; Keilp, J.; Rubinow, D.; Markey, S. P. *Ann. Neurol.* **1991**, *29*, 202.
(5) Kishore, G. M. *J. Biol. Chem.* **1984**, *259*, 10669.
(6) Phillips, R. S.; Dua, R. K. *J. Am. Chem. Soc.* **1991**, *113*, 7385.

0002-7863/93/1515-1264$04.00/0