Unfolding and breakdown of insulin in the presence of endogenous thiols

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Received 30 March 2005; revised 5 May 2005; accepted 3 June 2005

Available online 20 June 2005

Edited by Christian Griesinger

Abstract Native insulin denatures and unfolds in the presence of thiol catalyst via disulfide scrambling (isomerization). It undergoes two transient non-native conformational isomers, followed by an irreversible breakdown of the protein to form oxidized A- and B-chain. Denaturation and breakdown of native insulin may occur under physiological conditions. At 37 °C, pH 7.4, and in the presence of cysteine (0.2 mM), native insulin decomposes with a pseudo first order kinetic of 0.075 h^{-1}. At 50 °C, the rate increases by 5-fold. GdnCl and urea induced denaturation of insulin follows the same mechanism. These results demonstrate that stability and unfolding pathway of insulin in the presence of endogenous thiol differ fundamentally from its reversible denaturation observed in the absence of thiol, in which native disulfide bonds of insulin were kept intact during the process of denaturation.

Keywords: Insulin; Stability of insulin; Thermal denaturation of insulin; Unfolding of insulin; Isomerization of insulin; Structure of denatured insulin

1. Introduction

Insulin is a globular protein comprising two polypeptides, A-chain (21 residues) and B-chain (30 residues), cross-linked by three native disulfide bonds [1]. It is generated in vivo via proteolytic processing of a single chain precursor, proinsulin [2,3]. Insulin was the first protein produced by recombinant DNA technology for therapeutic application [4]. As one of the best characterized and most widely used therapeutic proteins, the data of insulin stability is of prime importance with respect to its structure–function, its formulation and administration. The covalent stability [5–7], polymerization [8,9], aggregation [10,11] and fibril formation [12–16] of insulin have been investigated most extensively. The conformational stability of insulin has also been studied by different laboratories [17–21]. Many of these data were obtained at different pH or salt concentrations or in the presence of an organic co-solvent and most investigations were conducted by the "*disulfide intact*" denaturation, in which the network of native disulfide bonds of insulin were kept intact during the process of denaturation.

Our laboratory has recently shown that conformational stability of a disulfide protein may decrease significantly in the presence of catalytic amount of free thiols, including endogenous amino-thiols (e.g., cystein (Cys) and reduced glutathione (GSH)) [22]. Under these conditions, the rigid network of native disulfide bonds is rendered flexible and denaturation of the protein is escorted by "*disulfide scrambling*" (disulfide isomerization) according to the conformation induced by the denaturant [23,24]. The denaturation results in the formation of a mixture of heterogeneous scrambled isomers, which are acid-stable can be trapped in acid, analyzed by high performance liquid chromatography (HPLC) and quantified with accuracy [24]. The extent of denaturation inflicted by the denaturant is directly reflected by the extent of conversion of the native protein to the scrambled isomers. For instance, the conformational stability of bovine pancreatic phospholipase A_2, $\Delta(\Delta G^{H20})$, is reduced by 6.2 kcal/mol when disulfide isomerization is allowed in the presence of thiol catalyst [22]. This phenomenon has been similarly observed in the cases of ribonuclease A [25,26] and α-lactalbumin [24,27].

Using the technique of "*disulfide scrambling*" denaturation, we have revisited the conformational stability of bovine insulin in the presence of endogenous thiols. The results show that denaturation of native insulin undergoes two previously identified conformational isomers and leads to an irreversible breakdown of the protein. It is demonstrated here that unfolding and breakdown of a significant fraction of native insulin may occur even at physiological pH, temperature and in the presence of physiological concentration of endogenous thiol.

2. Materials and methods

2.1. Materials

Bovine pancreatic insulin (I-5500) was obtained from Sigma. The protein was re-purified by HPLC with a final purity of greater than 99.8%. Cysteine, reduced and oxidized glutathione, urea and guanidine hydrochloride (GdnCl) were also purchased from Sigma, all with purity greater than 99.5%. Human serum was obtained from one of the authors (C.T. Jiang).

2.2. Denaturation of the native insulin in the presence of thiols

For thermal denaturation, the native insulin (0.5 mg/ml) was dissolved in the phosphate buffer (20 mM, pH 7.4, 100 mM NaCl) containing different concentrations of Cys (15, 50 and 200 μM) or GSH (50 and 200 μM). Denaturation was carried out at 37 and 50 °C for up to 24 h. In the cases of chemical denaturation, the native

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Abbreviations: HPLC, high performance liquid chromatography; GdnCl, guanidine hydrochloride; Cys, cysteine; GSH, reduced glutathione; GSSG, oxidized glutathione; TFA, trifluoroacetic acid

0014-5793/$30.00 © 2005 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2005.06.010

insulin (0.5 mg/ml) was incubated in the Tris–HCl buffer (0.1 M, pH 7.4) containing Cys (200 μM) and selected concentrations of urea and GdnCl. Denaturation was carried out at 22 °C for up to 24 h. To monitor the kinetics and intermediates of unfolding, aliquots of insulin sample were removed at different time points, quenched with an equal volume of 4% aqueous trifluoroacetic acid (TFA) and analyzed by reversed-phase HPLC.

2.3. Denaturation of insulin in human serum

Native insulin (0.5 mg/ml) was dissolved in an undiluted human serum. The serum samples were incubated at 37 and 50 °C for a time period up to 8 h. A serum sample without inclusion of insulin was processed as the control. Denatured insulin was quenched in a time-course manner by mixing aliquots of reaction samples with a double volume of 4% aqueous TFA, kept at room temperature for 30 min, followed by centrifugation at $16000 \times g$ for 10 min. The supernatant was filtered by passing through a 0.2 μm PVDF syringe filter (4 mm diameter). The filtrate was then directly analyzed by HPLC.

2.4. HPLC analysis of the denatured products of insulin

Denatured insulin samples were analyzed by HPLC using the following conditions. Column was Zorbax 300XB-C18, 250 mm × 4.6 mm 5 μm. Buffer A was 0.1% TFA in water. Buffer B was 0.086% TFA in acetonitrile/water (9:1, by volume). The gradient of elution was 20% B to 70% B linear in 40 min. The flow rate was 0.5 ml/min. Column temperature was 22 °C.

2.5. Chemical modification of the denatured products of insulin

Fractions of denatured insulin (~20 μg) were isolated, freeze-dried and modified with vinylpyridine (5 μl) or iodoacetic acid (0.1 M) in 50 μl of Tris–HCl buffer (0.1 M, pH 8.4). Reactions were allowed at 23 °C for 45 min, acidified with an equal volume of 4% aqueous TFA, and analyzed by RP-HPLC and MALDI-TOF.

2.6. Mass spectrometry analysis of the denatured products of insulin

The molecular mass of denatured products of insulin, both unmodified and those modified with vinylpyridine and iodoacetic acid, were determined by MALDI-TOF mass spectrometer (Perkin–Elmer Voyager-DE STR) using 2,5-dihydroxybenzoic acid as matrix. Molecular mass of analyzed peptides were calibrated by the following standards. Bradykinin fragment (residues 1–7) (MH^+ 757.3997); Synthetic peptide P14R (MH^+ 1533.8582) and ACTH fragment (residues 18–39) (MH^+ 2465.1989).

3. Results

3.1. Thermal denaturation of native insulin

Thermal denaturation of insulin was performed in the buffer (pH 7.4) containing Cys (0.2 mM) at both 37 and 50 °C for up to 24 h. Acid trapped samples were analyzed by HPLC. The results, given in Fig. 1, show that decrease of the native insulin, was accompanied by the appearance of two minor transient intermediates (fractions c and d), followed by the emergence of three major products (denoted as a1, a2 and b). Time-course denatured intermediates were also trapped by either vinylpyridine or iodoacetic acid and analyzed by the same HPLC system. The results are indistinguishable from the acid trapped sample shown in Fig. 1, suggesting that all five fractions of denatured insulin exist in oxidized form and none of them contains free cysteine. GSH catalyzed thermal denaturation of insulin follows the same unfolding pathway.

Fractions a1, a2, b, c, and d were isolated and analyzed by MALDI-mass spectrometry. Their observed molecular mass indicates that a1 (2336) and a2 (2336) are 2-disulfide isomers of A-chain (note that A-chain comprises two disulfide bonds and may potentially adopt three 2-disulfide isomers). Fraction b (3397) contains insulin B-chain with a disulfide bond linking

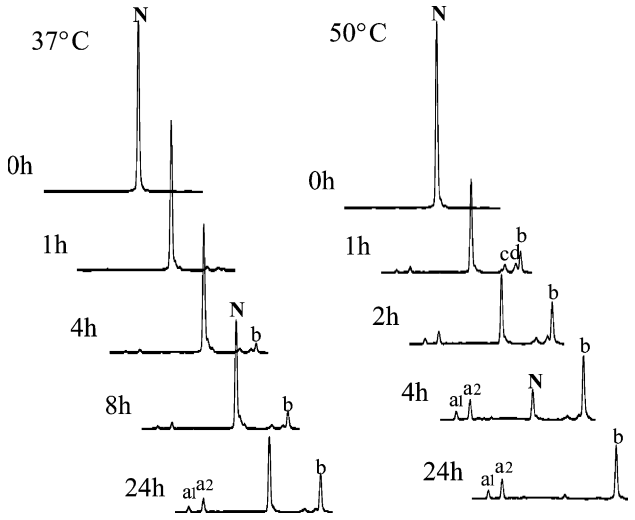

Fig. 1. Thermal denaturation of insulin in the presence of endogenous thiol. Insulin was incubated in phosphate buffer (20 mM, pH 7.4, 0.1 M NaCl) containing 200 μM of Cys. The protein concentration was 25 μg/50 μl. Denaturation was carried out at 37 and 50 °C for up to 24 h. Time-course incubated samples were quenched by acidification and analyzed by HPLC using the conditions described in the text. The decrease of native insulin (N) is accompanied by the appearance of two transient intermediates (c and d) which are isomers of N, followed by the increase of three fractions of end products (a1, a2 and b) which are oxidized insulin A- and B-chain.

Cys^{B7} and Cys^{B19}. Fractions c and d both exhibit molecular mass (5733) identical to that of native insulin, suggesting that they are non-native conformational isomers of intact insulin. The HPLC profile of fractions c and d are compatible with the two disulfide isomers of human insulin, designated as insulin-*swap*[1] and insulin-*swap*[2], identified previously [20,28,29]. Structures, stability and functions of these two insulin isomers have been well characterized by Weiss and co-workers [20,29].

Thermal denaturation of insulin results in an irreversible breakdown of the protein. At physiological pH, 37 °C and in the presence of Cys (0.2 mM), native insulin decomposes with a pseudo-first order kinetics of 0.075 h^{-1}. The rate of insulin breakdown increases by about 5-fold at 50 °C and decreases by 3.5-fold when a lower concentration of Cys (50 μM) was applied (Fig. 2). GSH is less effective than Cys in catalyzing disulfide isomerization of insulin, noticeably at 37 °C. The simultaneous presence of GSH/GSSHG (molar ratio 2:1), a condition that mimics the cellular environment, is also capable of promoting thermal denaturation of insulin.

3.2. GdnCl and urea denaturation of native insulin

Chemical denaturation of insulin was performed in the Tris–HCl buffer (pH 7.4) containing Cys (0.2 mM). The pathway of GdnCl and urea induced denaturation of insulin is shown to be indistinguishable from that of thermal denaturation. Unfolding of native insulin also undergoes two minor transitory intermediates, c and d, and ends-up with an irreparable breakdown of the protein to form the oxidized A- and B-chain (Fig. 3). In term of potency in denaturing insulin (measured by kinetics of denaturation), 3 M of GdnCl is roughly equivalent to 50 °C, with a pseudo-first order kinetic of 0.375 h^{-1} (Fig. 4A). 37 °C is as effective as 1 M GdmCl or 2 M urea. The potency of GdnCl is about 2-fold greater than urea as expected (Fig. 4B).